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NOTES

Microsatellite Multiplexing in Fish

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Abstract.—Microsatellite multiplexing is a powerful technique that can increase the productivity of genetic studies in fisheries biology. We review multiplexing methods and present an optimized and detailed protocol for microsatellite multiplexing that is specifically tailored for use with radioisotopes. The protocol can significantly reduce the cost associated with microsatellites and provides high polymerase chain reaction (PCR) fidelity and band resolution. Comparing three radioisotopes, we find that end labeling with ^{33}P provides the highest resolution. We also present a quick and inexpensive DNA isolation protocol that is successful with fish larvae. Finally, we find that PCR fidelity depends on the quality of the DNA template, and we therefore review preservation and isolation methods specific to various fish tissue types. Together, these microsatellite multiplexing and DNA isolation protocols can significantly reduce the time and expense associated with genetic analyses in fish.

Microsatellites are versatile genetic markers that are finding applications in many studies of ecology, evolution, and conservation (for reviews, see Wirgin and Waldman 1994; O'Reilly and Wright 1995; Jarne and Lagoda 1996; O'Connell and Wright 1997). In fisheries biology, these markers are widely used to assess the following: effective population size of stocks (Reilly et al. 1999), stock identification (Shaklee and Bentzen 1998), levels of inbreeding (Tessier et al. 1997), population structure and gene flow (DeLeon et al. 1997; Arnegard et al. 1999), parentage (Knight et al. 1998), and quantitative traits (Jackson et al. 1998). Polymerase chain reaction (PCR) multiplexing, the coamplification of two or more loci in a single PCR reaction (Chamberlain et al. 1988), is an innovative technique that considerably reduces the time and costs associated with microsatellite genetic analyses. However, many fisheries laboratories are not multiplexing because of the lack of effective and detailed protocols and because of the general apprehension that multiplexing considerably increases the complexity of using microsatellites.

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Although several multiplexing protocols exist for the fluorescent tags and automated detection systems (e.g., Edwards et al. 1991; Kimpton et al. 1993; Oetting et al. 1995; Paetkau et al. 1995; Ricciardone et al. 1997; Fishback et al. 1999), these systems are expensive and are unavailable to most fish laboratories. Instead, these laboratories use radioisotopes, polyacrylamide gel electrophoresis, and autoradiography to visualize microsatellites. The use of radioisotopes presents several additional complexities to multiplexing that the protocols tailored to fluorescent tags do not address. For example, microsatellite loci with alleles that overlap in size cannot be differentiated with radioisotopes. Further, the lack of internal size standards in individual multiplex reactions impedes accurate scoring of radioisotope-labeled banding patterns, thereby placing emphasis on resolution and visualization. Finally, existing radioisotope-based protocols (e.g., Huang et al. 1992; O'Reilly et al. 1996) are generally costly for large sample sizes, and they may produce inconsistent resolution, particularly when researchers are amplifying dinucleotide microsatellites.

This paper (1) reviews the key papers that discuss multiplexing; (2) presents a step-by-step protocol for the design and optimization of multiplexes specifically tailored for use with radioisotopes; (3) evaluates the performance of three radioisotopes; (4) evaluates the effect of DNA purity on multiplex fidelity; and (5) summarizes methods of preservation and isolation of DNA for different types of fish tissue. The step-by-step protocol provides a comprehensive set of procedures, including primer design, PCR coamplification, multiplex optimization, and electrophoresis and visualization.

Multiplex Protocol

We have found that the following protocols produce reliable microsatellite primers, amplified product, and allele band visualization. The multiplex protocol is summarized in Figure 1.

(1) Primer Design

To flank the microsatellite, ''forward'' and ''reverse'' primers are designed using the program

PRIMER (version 0.5) (Lincoln et al. 1991). The program parameters are set with an annealing temperature of 58°C, a length of 19–23 bases, and termination with a G or C (i.e., 3′ G/C clamp). Primer self-complementarity is restricted to 12 or fewer bases, and 3′ end primer self-complementarity is restricted to 8 or fewer bases. These parameters should minimize nonspecific binding of the primers and maximize PCR fidelity. Primers are designed such that the product lies in one of five target ranges: (1) 80–120 bases; (2) 121–160 bases; (3) 161–200 bases; (4) 201–240 bases; or (5) 241–280 bases. Several microsatellite loci are developed in each product size range so that many combinations can be tested for multiplexing.

(2) Single-Locus PCR

An initial screen of the microsatellites enables the researcher to select loci (for multiplexing) that are polymorphic, that have consistent amplification conditions (annealing temperature and $MgCl_2$ concentration), that have allele length distributions that do not overlap, and that have similar product intensity. The single-locus protocol is the same as the multiplex PCR protocol described below, except that it uses only a single primer pair.

(3) Multiplex PCR

Our DNA thermal cycler (MJ PTC-200 DNA Engine) is set at the following parameters: 60 s at 92°C; seven cycles of 30 s at 92°C, 30 s at 58°C, and 20 s at 72°C; and 28 cycles of 15 s at 92°C, 30 s at 58°C, and 20 s at 72°C. The 10-μL PCR reactions include 10–100 ng of total DNA, 10 mM tris-HCl (pH 8.3), 2.0–2.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM of each deoxynucleotide (Pharmacia; Piscataway, New Jersey), 10 μg bovine serum albumin (BSA; Pharmacia), 0.02 μM of each forward and reverse primer, and 0.25 units *Taq* DNA polymerase (Gibco BRL; Gaithersburg, Maryland). The reverse primer comprised unlabeled and end-labeled primer in a 1:1 ratio. A 10-μL end-labeling reaction includes 1× labeling buffer (New England Biolabs; Beverly, Massachusetts), 1 μM reverse primer, 1 μL of γ-^{33}P (10 mCi/mL, 2,000 Ci/mmole; Amersham; Oakville, Ontario), and 20 units of T_4 polynucleotide kinase (New England Biolabs) and is incubated at 37°C for 30 min followed by a 70°C incubation for 10 min. Gamma-^{35}S (10 mCi/mL, 1,000 Ci/mmole; ICN Pharmaceuticals) and γ-^{32}P (10 mCi/mL, 600 Ci/mmole; ICN) can be substituted for the ^{33}P with no change in the protocol.

(4) Electrophoresis and Visualization

The PCR product is mixed with one part stop dye deionized formamide and 100 mM EDTA), and 2–4 μL are hot-loaded (maintained at 95°C in a heating block) on a prewarmed 6% denaturing polyacrylamide gel containing 8.5 M urea. The gel is run at a constant power of 100 W, at approximately 2,000 V, at 50 mA, and a temperature of about 55°C. To visualize the separated PCR product, the gels are blotted, dried, and exposed to autoradiograph film (BIOMAX-MR KODAX) for 12–72 h. Exposure time is dependent on the intensity of the product and the isotope (e.g., longer times are required for ^{35}S).

DNA Isolations for PCR Analysis

We isolated DNA from tissue samples with one of two protocols. Protocol 1 is a simple proteinase-K digestion, whereas protocol 2 involves a more complex organic extraction following sodium dodecyl sulfate (SDS) and proteinase-K treatment (e.g., Bardakci and Skibinski 1994). In protocol 1, about 3 mm^3 of tissue is submerged in 50 μL of tris-EDTA (TE; 10 mM tris [pH 8.0], 1 mM EDTA) containing 500 μg/mL of proteinase-K (Gibco BRL). The samples are incubated for 18–24 h at 65°C (or for 2 h with continuous mixing). This is followed by incubation for 10 min at 95°C to denature any protein, including the proteinase-K. The samples are then centrifuged, and the supernatant is used directly in the PCR reactions. In protocol 2, about 25 mg of tissue is homogenized in lysis buffer (500 μL of TE, 30 μL of 1% SDS, and 30 μL of proteinase-K [10 mg/mL]) and then incubated at 65°C for about 2 h, with continuous mixing. After the incubation, two organic extractions are used: phenol–chloroform–isoamyl alcohol (25:24:1) followed by chloroform–isoamyl alcohol (24:1). Finally, the samples are ethanol precipitated, dried, resuspended in 50 μL TE, and used in the PCR reactions. For both protocols, samples that are preserved in ethanol are soaked in distilled water for 18–24 h prior to the isolation to remove the ethanol from the tissue.

Results and Discussion

Why Multiplex?

Even with the advent of preliminary multiplexing protocols and automated genotyping techniques, many laboratories still run single-locus reactions. This is due in part to the high cost associated with purchasing automated equipment and in part to researchers' reservations about the potential com-

Step 1: *Primer Design*

- 19 to 23 bases, 58°C annealing temperature, termination with G or C
- primer self-complementarity ≤ 12, 3' end primer self-complementarity ≤ 8
- amplified product length in 1 of 5 ranges: (1) 80-120 bases, (2) 121-160 bases, (3) 161-200, (4) 201-240 and (5) 241-280*
- use Primer or similar software

Step 2: *Single-locus PCR*

- amplify all loci individually
- use $MgCl_2$ concentration from 1.5 mM - 2.5 mM
- use annealing temperatures of 54°C - 62°C

Step 3: *Multiplex PCR*

- select loci with non-overlapping product size, similar PCR conditions and product intensity; alternatively, ensure that upper loci have equal or greater product intensity*
- use annealing temperature that gives good product intensity, but minimizes non-specific product amplification

Step 4: *Multiplex Optimization*

(a) All products are weak

- decrease annealing temperature by 2°C
- increase length of annealing and extension time
- increase $MgCl_2$ in PCR*
- increase number of PCR cycles*
- increase T_4 PNK in labelling reaction*
- increase exposure of autoradiograph*

(b) Short products are weak

- increase primer concentration of weak locus
- decrease annealing temperature by 2°C
- increase $MgCl_2$ in PCR*
- increase T_4 PNK in labelling reaction of weak locus*
- increase PCR buffer and KCl concentration to 1.4-2.0× (Henegariu et al. 1997)

(c) Long products are weak

- increase annealing temperature by 2°C
- decrease $MgCl_2$ in PCR*
- increase T_4 PNK in labelling reaction of weak locus*
- decrease PCR buffer and KCl concentration to 0.7-0.8× (Henegariu et al. 1997)

(d) Intense non-specific product appears

- purify DNA template*
- decrease amount of template
- increase annealing temperature by 2°C
- decrease $MgCl_2$ in PCR*
- try alternative combination of loci*

(e) One or more loci do not amplify*

- check all primers for interactions (e.g., complimentarity)
- increase primer concentration of missing locus
- increase annealing temperature by 2°C
- increase/decrease $MgCl_2$ in PCR
- try alternative combination of loci

(f) Products are smeared and fuzzy*

- increase urea concentration in gel
- increase amount of stop dye added
- hot load samples
- load less sample onto gel
- dry blot gel (i.e., do not fix gel)
- decrease number of PCR cycles
- try labelling with ^{33}P

(g) Dull-grey background on autoradiograph*

- fix gel with 10% methanol and 10% acetic acid; wash gel thoroughly with fixer
- purify DNA template (e.g., ensure residual ethanol and phenol are removed from sample)
- try labelling with ^{33}P

(h) Dark streaks appear on autoradiograph*

- dry blot gel (i.e., do not fix gel) or fix for longer time
- dry gel more thoroughly
- lightly coat gel with drying agent (e.g., baby powder)

plexity and product uncertainty of multiplexing. The present multiplex protocol provides a simple and effective means for reducing costs and time without forfeiting genotyping consistency and band resolution (Figure 2a). For example, multiplexing four loci requires four times less the amount of PCR consumables (e.g., tubes, *Taq* polymerase, and other reagents), polyacrylamide gels, and film. Multiplexing can increase genotyping productivity with relatively simple adjustments to existing protocols.

Advantages of the Present Protocol

The present optimized multiplex protocol provides several modifications (Table 1). It greatly improves band resolution by reducing the occurrence of secondary structures that cause unclear bands. This is accomplished in three ways. First, excessive unlabeled PCR product is significantly reduced by decreasing the primer concentration by up to sixty-fold. However, band intensity is not compromised since proportionately there is a five-fold increase in labeled product. Further, excess unlabeled primer of other protocols may competitively exclude labeled primer, and consequently, most of the PCR product is unlabeled and not visualized. Second, ''hot-loading'' the samples facilitates the denaturation of double-stranded DNA prior to electrophoresis. Third, increasing the concentration of the denaturant urea in the polyacrylamide gel promotes the maintenance of a linear conformation of single-stranded DNA during electrophoresis.

The present protocol also increases product yield and reliability by adding BSA to the PCR reactions (Innis et al. 1988; Henegariu et al. 1997; Stommel et al. 1997). Furthermore, it is more cost effective since it utilizes lesser amounts of isotope, primer, and *Taq* DNA polymerase. For example, it uses as much as 19 times less isotope and four times less *Taq* (Table 1)—the principal costs of PCR-related consumables.

Multiplex Optimization

Examples of sequences and design characteristics of four primer sets are presented in Table 2. Generally, the Primer Design protocol provides primers that are reliable and produces minimal nonspecific amplification. Although all of our primers terminate in a G or C, we have used others that terminate in an A or T with similar success. However, G/C–rich sequences are more thermally stable, and therefore, primers terminating in these bases should provide a more reliable priming site during PCR.

We have found that most combinations of primer sets work well together, and we routinely coamplify several combinations of up to five loci (Table 3). In total, 11 of 15 loci were successively multiplexed in several combinations, which suggests that researchers following the present protocols may not need to isolate a large number of loci in order to generate their multiplexes.

Although most combinations of loci work well with no adjustment to primer concentration or to other amplification conditions, some require minor modifications to the protocol (see Figure 1). For example, the lowest locus (i.e., shortest alleles), which *Taq* DNA polymerase can preferentially amplify, may appear too intense in the multiplex, whereas the highest locus may appear too faint. In this case, the primer concentration of the lowest locus can be halved (0.01 μM of each primer) to reduce its intensity, and the primer concentration of the upper locus can be doubled (0.04 μM of each primer) to increase its intensity. Generally, the best multiplex results are obtained when the upper loci (i.e., longer PCR products) have equal or greater product intensity when comparing the single-locus reactions. The choice of whether to end label the reverse primer is arbitrary. We find that labeling either primer generates similar results; however, differences may exist with some loci (see O'Reilly et al. 1996). The following modifications contributed minimally to consistency and band resolution and were not considered effective (also see Henegariu et al. 1997): (1) increasing the PCR reaction volume from 10 to 20 μL; (2) increasing the number of PCR cycles; (3) increasing the annealing or extension time; (4) increasing the concentration of the dNTPs; (5) increasing the amount of DNA template; and (6) increasing the amount of *Taq* DNA polymerase. Although we find that doubling the concentration of the PCR buffer (specifically KCl) increases efficiency only modestly, it can reduce differential

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FIGURE 1.—Step-by-step protocol for the design and optimization of microsatellite multiplexing (modified from Henegariu et al. 1997). Asterisks indicate parameters not tested by Henegariu et al., and references to them indicate parameters not tested here. All other parameters were tested by both.

amplification in heterozygotes (Fishback et al. 1999) and improve band intensity of product visualized on agarose gels (Henegariu et al. 1997).

Primer sets with different optimal annealing temperatures can be multiplexed using touchdown PCR. Touchdown PCR temperature profiles begin with an elevated annealing temperature and systematically reduce it by typically 0.5°C during each cycle until the desired lower annealing temperature is obtained (see Rithidech et al. 1997). Fishback et al. (1999) find that touchdown PCR enables the coamplification of loci with different optimal annealing temperatures without the production of artifact bands.

As an alternative to end labeling, we find that direct incorporation of the radioisotope (e.g., α-dATP) into the PCR reduces consistency and resolution. This may be due in part to the labeling of complementary DNA strands that have different sequences and slightly different mobility during electrophoresis. The subsequent visualization of both complementary DNA strands can decrease the resolution and impede genotype identification. For this reason, we find that end labeling, whereby only one strand is labeled and visualized, is the more effective approach.

Adenylation can occur during the PCR amplification of some microsatellites, and it causes an increase of one base in allele sizes. Variation in the degree of adenylation can decrease reproducibility and impede accurate allele scoring. Brownstein et al. (1996) showed that the degree of adenylation could be controlled by redesigning the reverse primers and by altering the PCR temperature profile. Fishback et al. (1999) found that decreasing primer and increasing *Taq* polymerase concentrations resulted in consistent adenylation of alleles without the need to redesign primers. We also find that the similar reduced primer concentration in our protocol provides consistent results. However, we find that adherence to a single PCR temperature profile provides reproducible results without the need to elevate the concentration of costly *Taq* polymerase (also see Olsen et al. 1996).

Allele length distributions are generally wider for fish microsatellites compared with those of mammals (O'Reilly et al. 1996; authors' unpublished data). For example, we have found that in fish, allele length distributions of dinucleotide microsatellites average about 40 bases and are nearly twice that of mammals (authors' unpublished data). As such, the targeted product size range for multiplexing of fish microsatellites must be wider to ensure that alleles do not overlap. This can limit the number of microsatellites that can be multiplexed, since the effective range of separation using standard denaturing polyacrylamide electrophoresis is about 200 bases. Therefore, on average, fish multiplexes may be limited to about five loci.

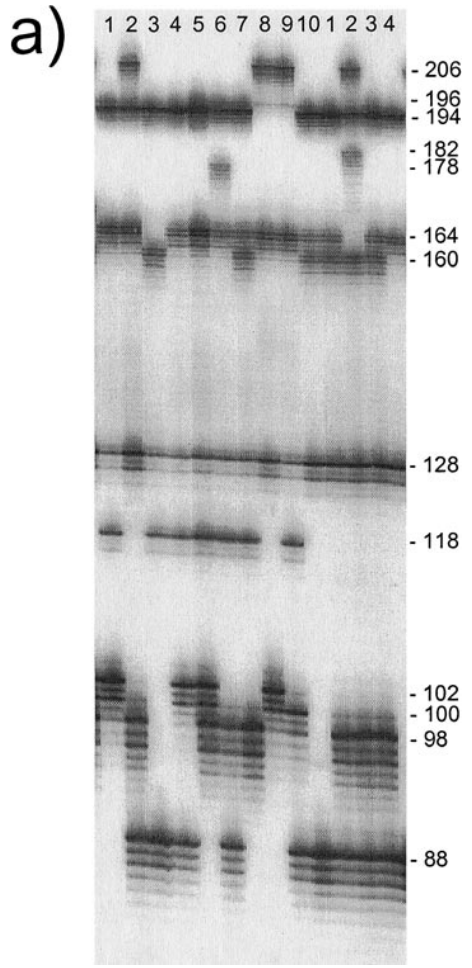

FIGURE 2.—Multiplex polymerase chain reactions (PCRs) of loci isolated from bluegill sunfish *Lepomis macrochirus*. The loci are Lma102, Lma87, Lma21, and Lma117 labeled with **(a)** ^{33}P, **(b)** ^{32}P, and **(c)** ^{35}S. All individuals were fry (fish larvae) from a natural nest. The stutter, typical of dinucleotide loci, is no more prevalent than that in single-locus reactions. Allele lengths are indicated, and the loci do not overlap. The genotypes are easily identified following the methods of O'Reilly and Wright (1995) (note the sequence ladder is not shown). For example, the genotypes of the first individual in **(a)** are 102/102, 118/128, 164/164, and 194/194. All primer sequences appear in Colbourne et al. (1996) and Neff et al. (1999).

Although we primarily used microsatellites with dinucleotide repeats (dimers), we have also tested

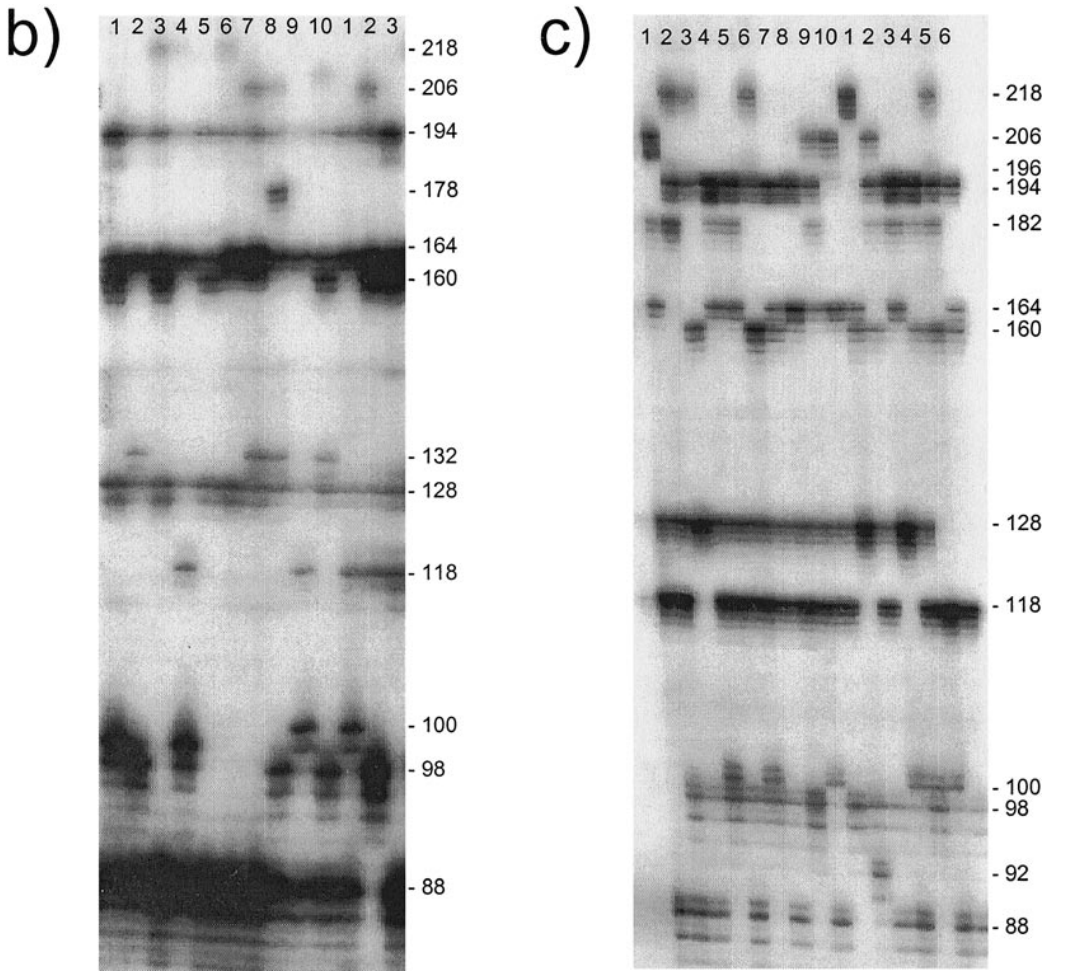

FIGURE 2.—Continued.

TABLE 1.—Summary and comparison of the key components of three multiplex protocols. Abbreviations are BSA = bovine serum albumin; PCR = polymerase chain reaction; UN = unknown.

Component	Huang et al. (1992)	O'Reilly et al. (1996)	Present study	Present protocol	
				Comments	Benefits
Primer (μM)	0.06–0.60	0.15–0.50	0.01–0.04	Generates less PCR product and decreases occurrence of secondary structure	Increases band resolution
Taq polymerase per genotype (units)	1.0	0.50	0.25	Requires less *Taq* polymerase	Reduces costs
Ratio of labeled : unlabeled	N/A	1:9	1:1	Increases proportion of PCR product visualized	Maintains band intensity with less PCR product
Isotope per genotype (μCi)	0.2–1.9	0.1–0.34	0.1–0.2	Requires less isotope	Reduces cost
BSA in PCR mixture	No	No	Yes	Stabilizes *Taq* polymerase	Increases PCR fidelity and band resolution
Gel urea (M)	7.0	7.8	8.5	Decreases occurrence of secondary structure	Increases band resolution
Hot loading of samples	No	UN	Yes	Promotes denaturation of double-stranded PCR product	Increases band resolution

TABLE 2.—Design characteristics of four microsatellite primer sets isolated from bluegill sunfish. The primer characteristics include nucleotide sequence, length, G/C content (% G/C), melting temperature (T_m), number of terminating bases of G or C (3′ G/C), primer self-complementarity, and the allele size range.

Locus	Primer′ sequence (5′–3′)[a]	Length (bases)	% G/C	T_m (°C)	3′ G/C (bases)	Primer self-complementarity: Entire	Primer self-complementarity: 3′ End	Size (bases)[b]
Lma102[c]	F: CTGTGAAAATGGTGTGAGCG	20	50	60.3	3	2	2	88–102
	R: AAACACAAAAGTCCACGCAC	20	45	58.7	1	2	2	
Lma87[d]	F: ATGACACAGACTCACCATGC	20	50	56.9	2	4	3	118–152
	R: CTCCTGCCCATAAATCAGAC	20	50	57.2	1	3	2	
Lma21[d]	F: CAGCTCAATAGTTCTGTCAGG	21	48	55.2	2	5	4	158–182
	R: ACTACTGCTGAAGATATTGTAG	22	36	48.4	1	4	4	
Lma117[c]	F: CCACCAACAGCATGCAGAC	19	58	61.3	1	6	4	194–218
	R: CATGCCACTCATTGCACTG	19	53	59.8	1	6	6	

[a] Forward (F) and reverse (R).
[b] Allele size range based on 20 individuals (see Neff et al. 1999).
[c] Primer sequences previously reported in Neff et al. (1999).
[d] Primer sequences previously reported in Colbourne et al. (1996).

tetranucleotides (tetramers) with our protocols. We found that tetramers provided similar or better results than the dimers. For example, the PCR amplification of tetramers tended to be more consistent and produced fewer stutter bands that enhanced resolution and genotype identification (data not shown, but see O'Reilly et al. 1996). However, our tetramers were less polymorphic than the dimers, probably because they had fewer repeats (data not shown). Further, tetramers are less common in fish genomes (O'Reilly et al. 1996) and therefore can be difficult to isolate and develop. If polymorphic tetramers are available, they can be easily and effectively incorporated into the multiplex protocols.

Which Isotope?

In our laboratory, ^{33}P is the preferred isotope (over ^{32}P and ^{35}S). ^{32}P is the strongest emitter with the shortest exposure time (8–16 h). However, it has the greatest scattering property, which reduces band resolution (Figure 2b) and which can increase the variance in band intensities among loci. ^{35}S is the weakest emitter, but it generates crisp bands (Figure 2c). However, it requires extensive exposure time (up to 72 h) and is also the most volatile isotope. ^{33}P combines the best qualities of the other two isotopes. It has a relatively short exposure time (15–24 h) and generates crisp bands with little background (Figure 2a). Although ^{33}P is currently more expensive (about three times the cost of ^{32}P and ^{35}S), it increases productivity, accuracy of allele identification, and safety.

Which DNA Isolation Protocol to Use?

The effectiveness of techniques in isolating genomic DNA from fish species depends on both the preservation method and the tissue type (Table 4). For example, as ethanol-preserved tissues are more resistant to protease digestion by enzymes such as pronase (Taggart et al. 1992), such samples should be soaked in distilled water prior to DNA extraction, and a more active protease, such as proteinase-K, should be used. Furthermore, some fish species have powerful cellular endonucleases that prevent DNA extraction (Asahida et al. 1996). Therefore, inactivation of these enzymes during the preservation process can improve the quality of the extracted DNA. Asahida et al. (1996) have proposed the use of a preservation media containing a high

TABLE 3.—Microsatellite multiplexes for bluegill sunfish.

Size range[a]	Multiplexed loci[b]						
>240	Lma116			Lma116			
201–240	Lma113	Lma117	Lma113	Lma120	Lma113	Lma117	Lma113
161–200	Lma121	Lma21	Lma21	Lma121	Lma21	Lma21	Lma24
121–160	Lma117[c]	Lma87	Lma87	Lma122			
80–120	Lma20	Lma102	Lma102		Lma20	Lma20	Lma20

[a] Approximate size distribution of alleles.
[b] All primer sequences except Lma113 appear in Colbourne et al. (1996) or Neff et al. (1999).
[c] Alternative primer set for Lma117 (short; see Neff et al. 1999).

TABLE 4.—A summary of tissue preservation and DNA extraction and purification techniques.

Tissue type	Tissue preservation	DNA extraction	DNA purification	Comments
Blood	(1) Ethanol	(i) SDS and proteinase-K[a,b]	(a) Organic extraction[a]	The combination of (i) and (a) is common; however, organic solvents are toxic and can degrade DNA
		(ii) Triton X-100 and proteinase-K[c]	(b) Protein salting-out[d]	(b) may be a nontoxic alternative to (a), and provides high-quality DNA without additional contaminants
		(iii) Chelex resin[e]		(ii) may be used as a one-step process without the need for DNA purification
Fin, muscle or organs	(1) −20°C (2) Ethanol (3) Urea buffer[f]	(i) SDS and proteinase-K[a,b] (ii) Sarcosyl and pronase[g] (iii) Chelex resin[e]	(a) Organic extraction[a] (b) Protein salting-out[d] (c) Magnetic beads[h]	Ethanol reduces protease activity; ethanol-preserved samples should be soaked in distilled water prior to DNA extraction (3) may be superior to (1) and (2) as urea is both an inhibitor of cellular endonucleases and an activator of the proteinase-K Buffer used in (3) can be directly used as the lysis buffer in (i) Proteinase-K in (i) has greater activity than pronase in (ii) and may yield higher quality DNA, especially for ethanol-preserved samples; however, it costs about three times more Compared to the consistency of (i), (ii) is variable with different fish species (most effective with salmonid tissue) (c) is a new technique that is faster (<30 min/batch of samples) and provides comparable yields to (a); however, it is more expensive at about US$1/sample plus equipment cost
Fish or larva	(1) −20°C (2) Ethanol	(i) Proteinase-K[b] (ii) Chelex resin[e]	Optional	Ethanol-preserved samples should be soaked in distilled water prior to isolation (see above) (i) is a quick one-step process that generates DNA of sufficient quantity and quality for PCR analysis If higher quality DNA is required (e.g., see Figure 1), purification steps can be added If DNA yield is too low, consider GeneRelease kit (BioVentures)[i]
Fertilized eggs or larva with yolk sac	(1) −20°C (2) Ethanol	(i) Proteinase-K[b] (ii) Chelex resin[e]	Optional	Yolk containing contaminants such as endonucleases has to be separated from the developing embryo or larva prior to DNA extraction If eggs or larvae are too small for dissection, DNA purification steps are recommended to increase purity and to minimize degradation If DNA yield is too low, consider GenRelease kit (BioVentures)[i]

[a] Bardakci and Skibinski (1994).
[b] This study.
[c] Grimberg et al. (1989).
[d] Martínez et al. (1998).
[e] Estoup et al. (1996).
[f] Asahida et al. (1996).
[g] Taggart et al. (1992).
[h] Dynal (1996); Rudi et al. (1997).
[i] Schizas et al. (1997).

concentration of urea, one that not only inhibits cellular endonucleases but that also activates the proteinase-K used in the subsequent DNA extraction. This may be a good alternative to ethanol preservation, as DNA is protected from excessive cleavage during long-term storage. Finally, Taggart et al. (1992) found that adipose fin and liver tissue stored frozen for more than 1 year yielded

DNA that was more likely to be degraded than that yielded from skeletal muscle tissues preserved under similar conditions. Therefore, skeletal muscle may be the better tissue type for long-term preservation.

The trade-off between DNA quality and processing rate should also be considered in choosing an isolation protocol. Although PCR amplification of microsatellites is relatively robust to DNA template degradation because microsatellites are small, DNA purity can affect the success of PCR amplification, as contaminants can interfere with the polymerase. Furthermore, as the overall purity and quantity of DNA decreases as a result of contamination and excessive degradation, longer alleles may fail to amplify, and artifact bands may become more prevalent (also see O'Reilly et al. 1996). Fishback et al. (1999) found that increasing the concentration of poor-quality DNA (up to 1,500 ng per 15-μL reaction) can ensure amplification of all alleles but that additional optimization of primer concentrations may be required. Multiplex PCR reactions are particularly susceptible to DNA quality, as they have a wider range of allele sizes than reactions that amplify a single locus. However, the isolation of high-quality DNA comes at a price and is not always necessary. For example, protocol 2 (see above) produces higher quality DNA but requires about 10 times more contact time than does the simple proteinase-K digestion (protocol 1). We found that DNA isolated from tissues such as those of fish larvae (fry), in which the majority of tissue is used in the extraction process, amplifies consistently with the proteinase-K digestion, whereas DNA from other tissues, such as those of adult fish, typically require the more extensive protocols.

Summary

Many laboratories continue to run single-locus microsatellite reactions that are expensive in terms of the time and materials they require. The optimized microsatellite multiplex protocol presented here is easy to implement and can significantly reduce time and material costs. We hope it will allow the many laboratories employing radioisotope-based genotyping techniques to improve their research output.

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