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ARTICLE

The Use of Fluorescent Randomly Amplified Polymorphic DNA Markers to Identify Hybrids: A Case Study Evaluating the Origins of Saugeye following the Cessation of Stocking in an Ohio Reservoir

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Abstract
Hybridization and introgression continue to gain recognition as important issues in the management and conservation of native fishes. It is often necessary to identify hybrids in natural populations and to distinguish among individuals of various hybrid categories. Molecular methods are important for these purposes, and it is valuable if researchers have a range of molecular methods to apply, since each method has unique advantages and disadvantages. The determination of the best class of marker for a particular study depends on various factors, including the goals of the study, the resolution required, and the genomic and marker information already available for the taxa of interest. We modified a protocol to generate fluorescent randomly amplified polymorphic DNA (FRAPD) markers for hybridization studies. To our knowledge, this type of marker has not previously been used for hybrid identification. To demonstrate the utility of the modified methods, FRAPD markers were used to evaluate potential reproduction by saugeye (female walleye *Sander vitreus* × male sauger *Sander canadensis*) in a central Ohio reservoir. Our approach successfully generated a battery of diagnostic genetic markers that were used to test the hypothesis that young-of-year saugeye were later-generation offspring of saugeye cohorts previously stocked into the reservoir. Alternatively, the fish may have been immigrant first-generation saugeye from other sources. Data obtained from the FRAPD markers provided strong support favoring the alternative hypothesis. These methods provide a very useful tool for distinguishing between pure parentals and various classes of hybrid individuals, both in *Sander* spp. and in other taxa, offering a powerful and easily developed alternative to other molecular methods of generating informative genetic markers for hybridization studies.

Hybridization and genetic introgression are important factors to consider in the management and conservation of native fishes (Epifanio and Nielsen 2000; Laikre et al. 2010; Sato et al. 2010). In turn, the need for powerful and efficient methods for identifying hybrids, and for distinguishing among various hybrid categories, continues to grow. Molecular methods can provide much more power for identifying hybrid individuals than morphological approaches. This is especially true when

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hybridization extends beyond the first generation. Allozymes were one of the first types of molecular markers applied to questions of hybridization, and they continue to be used today (Echelle and Echelle 1997; Pierce and Van den Avyle 1997; Graeb et al. 2010). However, the number of informative allozyme loci is usually limited. More recently, DNA microsatellite loci have served as a good option in some hybridization studies (Hänfling et al. 2005; Cordes et al. 2006; Walters et al. 2008; Nolte et al. 2009; Ludwig et al. 2009). Nevertheless, because microsatellite markers are often taxon-specific, they may not be readily available for the study of many hybridizing groups. When they are available, assignment of individuals to various hybrid categories may be affected by both the potential occurrence of unidentified null alleles and an increased possibility of allelic homoplasy because of recurrent mutation. These factors are especially relevant when trying to distinguish among post-F_1 hybrid categories.

Dominant markers, which differ from codominant markers such as microsatellites in that they express a maximum of two alleles per locus (scored as present or absent), have also been utilized to identify hybrid individuals (Williams et al. 1998; Congiu et al. 2001; Yamazaki et al. 2005; Albert et al. 2006; Chelomina et al. 2008). Some dominant markers have the advantage of providing data from larger numbers of informative loci relative to most types of codominant markers. The two methods most often used for generating dominant molecular markers have been amplified fragment length polymorphism (AFLP; Vos et al. 1995) and randomly amplified polymorphic DNA (RAPD; Williams et al. 1990). The RAPD markers require less time and lower cost to generate than AFLP markers. However, concerns regarding the repeatability of RAPD markers have been raised (Jones et al. 1997; Pérez et al. 1998; Bagley et al. 2001). In this study, we applied three modifications to a traditional RAPD protocol that alleviate some of the concerns. We propose this modified RAPD methodology as an additional tool for consideration when developing loci for the genetic detection of hybrid individuals.

To demonstrate the effectiveness of our methods, the modified RAPD markers were used to evaluate alternative hypotheses concerning the origin of a population of hybrids in a central Ohio reservoir. Specifically, we generated a battery of genetic loci that allowed us to determine whether young-of-year saugeye (female walleye *Sander vitreus* × male sauger *Sander canadensis*), collected in Dillon Lake, Ohio for 3 years (2006–2008) during which no saugeye were stocked directly into the lake, were later-generation hybrid offspring of cohorts stocked prior to 2006. Alternatively, young-of-year saugeye may have been F_1 saugeye immigrants to the lake.

METHODS

Sample Collection.—Pectoral fin clips were collected from individuals representing 2005–2008 Ohio walleye and sauger broodstock populations ($N = 34$ and 30, respectively), known first-generation hybrid saugeye (hatchery-raised fingerlings from both Ohio and Indiana; $N = 40$), and from 12 age-0 or age-1 Dillon Lake individuals of unknown origin (see Table 1 and Figure 1 for sample locations). Upon collection, all samples were preserved in 70% ethanol.

TABLE 1. Sample locations and sample sizes from which tissue was obtained for genetic analyses.

Sample type and location	*N*
Walleye broodstock	
Berlin Lake	13
CJ Brown Reservoir	6
Findlay Lake	3
Maumee River	4
Mosquito Lake	6
West Branch Reservoir	2
Sauger broodstock	
Ohio River–Hannibal pool	4
Ohio River–Markland pool	14
Ohio River–McAlpine pool	4
Ohio River–Meldahl pool	8
F_1 Hybrid saugeye	
East Fork State Fish Hatchery	17
Hebron State Fish Hatchery	6
Senecaville State Fish Hatchery	7
St. Mary's State Fish Hatchery	10
Unknowns	
Dillon Lake	12

Extraction and Amplification of DNA.—Genomic DNA was extracted from fin clips using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Valencia, California), quantified with an Eppendorf BioPhotometer (Eppendorf North America, Westbury, New York), and diluted to a 40 ng/uL concentration. The PCR amplifications were performed on all samples with eight 10-base oligonucleotide primers originally designed for RAPD analyses by Eurofins MWG Operon, Huntsville, Alabama (primer sequences shown in Table 2). Each primer was labeled with a unique fluorescent tag (FAM, NED, PET, or VIC) on its 5′ end (fluorescently labeled RAPD, or FRAPD; Corley-Smith et al. 1997). The PCR reactions were carried out in 12.5 μL volumes and consisted of 1X ThermoPol reaction buffer, 0.5 units ThermoPol Taq DNA polymerase (New England Biolabs, Ipswich, Massachusetts), 1.5 nmol each dNTP, 10 pmol primer, and ~40 ng genomic DNA. The PCR cycling conditions consisted of 35 cycles of 94°C for 20 s, 46.5°C for 25 s, and 72°C for 1 min, with a decrease in annealing temperature of 0.3°C per cycle. These 35 cycles were followed by an additional 10 cycles of 94°C for 20 s, 35°C for 25 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were pooled for each sample in a 1:2:2:2 ratio (FAM:VIC:PET:NED) and electrophoresed on an Applied Biosystems 3730 genetic analyzer (Applied Biosystems, Carlsbad, California). The PCR reactions and electrophoresis were replicated for 29 of the 116 individuals (25%) in order to evaluate repeatability at the loci.

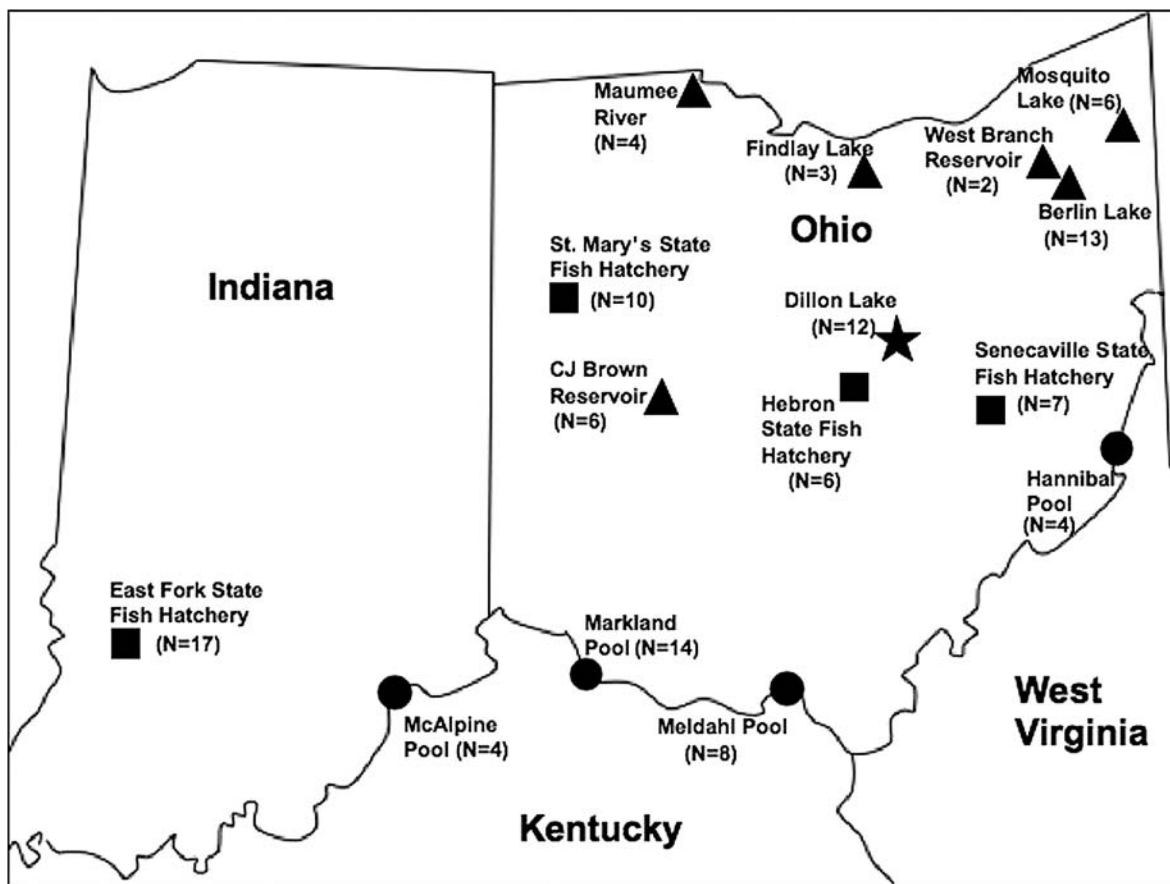

FIGURE 1. Sampling locations at which fin clips were collected representing walleye broodstock (triangles) and sauger broodstock (circles) used in Ohio saugeye production, known F_1 hybrid saugeye (squares), and individuals of unknown genetic ancestry (star). Broodstock populations used in the production of the East Fork State Fish Hatchery saugeye were from Brookville Reservoir in Indiana (walleye) and the Illinois River near Peru, Illinois (sauger).

Locus Identification and Scoring of Data.—The PCR products were analyzed for the presence or absence of specific bands corresponding to genetic loci in each individual using Genemapper version 3.7 genetic analysis software (Applied Biosystems,, Carlsbad, California). Anonymous genetic loci amplified in the PCR reactions were represented in Genemapper by a series of peaks and distinguished from each other by the size (in nucleotides) of the amplified products (Figure 2).

Individual loci for use in analyses were defined by bins approximately 1–2 basepairs in width, initially constructed by Genemapper, and then edited by hand. Genotype data were screened for any DNA fingerprints that appeared to have failed (those with especially low peak heights relative to the average along at least part of the fingerprint, or without any peaks at all). Such samples were rerun. For each sample, all peak heights with measured intensities of at least 50 units that

TABLE 2. Oligonucleotide primers used in PCR reactions (primer sequences designed by Eurofins MWG Operon, Huntsville, Alabama).

Primer name	5′ Fluorescent label	Output color	Sequence
OPM-1	FAM	Blue	5′GTTGGTGGCT
OPM-5	FAM	Blue	5′GGGAACGTGT
OPM-6	NED	Black/yellow	5′CTGGGCAACT
OPM-10	PET	Red	5′TCTGGCGCAC
OPM-11	VIC	Green	5′GTCCACTGTG
OPM-12	VIC	Green	5′GGGACGTTGG
OPM-13	NED	Black/yellow	5′GGTGGTCAAG
OPM-14	PET	Red	5′AGGGTCGTTC

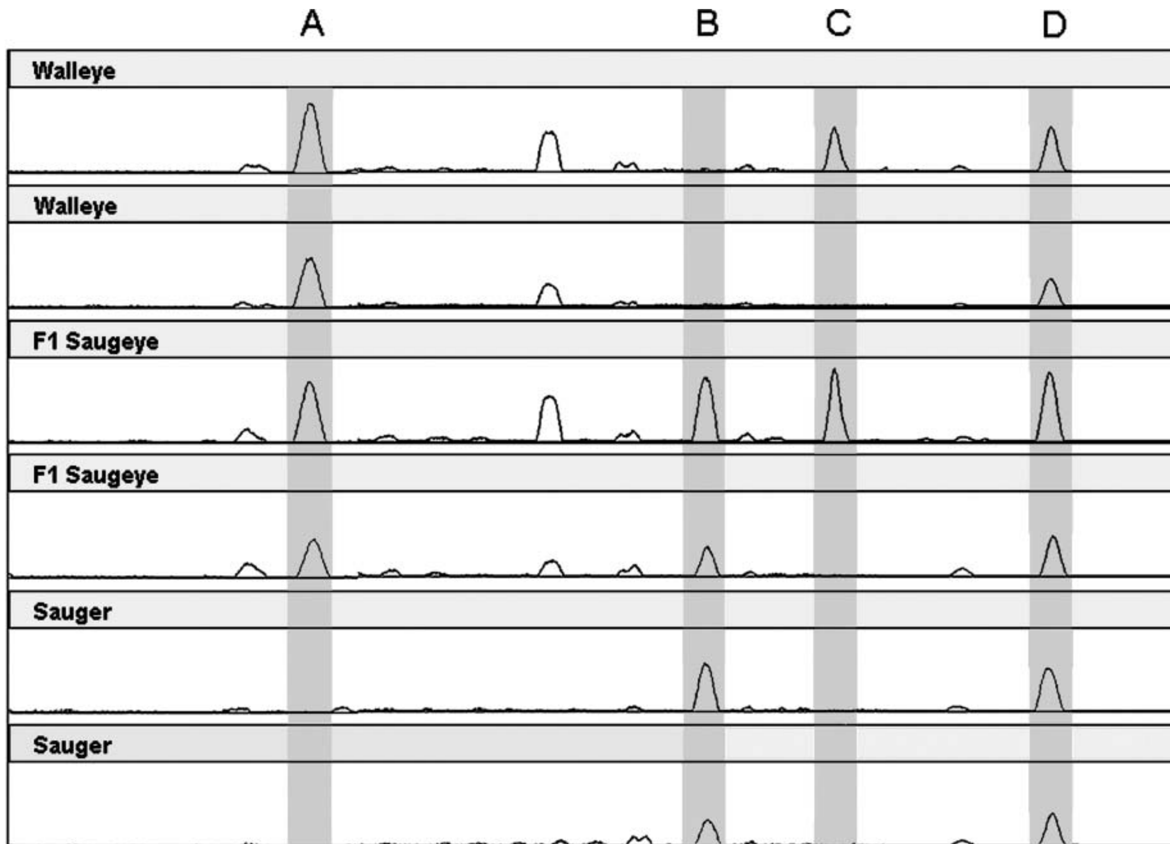

FIGURE 2. Example FRAPD data from six individuals. Four of the 63 scored loci are indicated by the shading. Peaks represent the presence of at least one allele in the individual at the locus. Loci labeled "A" and "B" are diagnostic for walleye and sauger, respectively. Locus "C" is informative (polymorphic in walleye, and absent in sauger). Locus "D" is an example of an uninformative locus.

occurred within the defined bins were obtained from Genemapper. These peak height scores were normalized in the R program AFLPScore according to the procedure described by Whitlock et al. (2008) for AFLP data. This normalization accounts for variability in factors such as sample dilution, PCR success, or PCR product dilution. The program then applies a filter to remove probable noise peaks, and allele scores (1 or 0, for presence or absence, respectively) were assigned based on a relative phenotype-calling threshold value of 0.1.

Loci were evaluated for repeatability based on samples from 29 randomly selected individuals which were run in replicate, starting from the PCR (a subset of the replicate samples was included on each sequencing run to serve as both replicates and positive controls). Only loci at which no mismatches were observed (100% repeatability) across all replicated samples were retained for analysis in the saugeye case study. These loci were used to estimate allele frequencies in the broodstock populations based on data from a combination of the broodstock samples and the saugeye obtained directly from the hatcheries (known to be F_1 hybrids). The allele frequency estimates were used to classify each locus into one of three classes that describe the utility of the locus in addressing questions of hybridization in *Sander* spp. Uninformative loci are those in which an allele peak is expressed in all individuals sampled, both from the broodstock populations and from the known hybrids. Informative loci are those that are polymorphic in at least one of the two broodstock populations. Diagnostic loci include those loci for which the "present" allele is present in all individuals in one of the broodstock groups, is completely absent in the other broodstock group, and is present in all known F_1 individuals. This third class contains the most highly informative loci with respect to questions of hybridization.

The loci designated as diagnostic were used to assign saugeye collected from Dillon Lake as either F_1 or later-generation hybrids. The F_1 individuals are expected to express the presence of a band peak across all diagnostic loci, whereas post-F_1 hybrids are expected to express the presence of a band peak at some, but not all, of the diagnostic loci. For example, an F_2 hybrid is expected to be homozygous for the "presence" allele at 25% of the loci, heterozygous at 50% of the loci, and homozygous for the "absence" allele at 25% of the loci. This leads to the expectation that F_2 individuals would express a peak at 75% of the diagnostic loci overall.

RESULTS AND DISCUSSION

In this study, we applied a series of modifications to a traditional protocol for generating RAPD genetic markers for hybridization studies. The first modification was the application of fluorescent tags to the oligonucleotides that were used as primers in the PCR reactions (Corley-Smith et al. 1997). These fluorescent tags allowed the amplified products to be scored on an automated DNA sequencer. This provided much greater resolution than previous methods, which have usually involved visualizing amplified products after electrophoresis in agarose gels. The second modification addressed potential subjectivity that can occur when scoring individual dominant markers. We employed the automated scoring methods described by Whitlock et al. (2008) to assign scores for the presence or absence of peaks for each individual at each genetic locus. This approach not only makes the process of allele-calling less subjective but also includes a normalization procedure that accounts for a number of factors that can introduce variability into dominant marker datasets. These factors can include variation in properties such as initial sample concentrations and PCR success between reactions. Third, PCR amplifications were performed using a touchdown PCR approach. The range of annealing temperatures utilized in touchdown PCR can help mitigate the effects of slight variability in parameters such as the block temperature between different thermocyclers. As a result, this modification may help to address concerns previously raised regarding the levels of repeatability associated with RAPD marker generation (Jones et al. 1997; Pérez et al. 1998; Bagley et al. 2001).

In order to illustrate the effectiveness of these modified methods in generating genetic markers that are useful for addressing questions of hybridization, we present the results of a case study which aimed to determine whether reproduction is occurring in a population of saugeye in Dillon Lake, Ohio. Using just eight FRAPD primers, we identified and scored 91 loci from each of 116 individuals prior to the screening of replicate samples. Evaluating repeatability in 29 replicate samples, the number of mismatches observed at each locus ranged from 0 to 5, although loci with larger numbers of mismatches were rare (Figure 3). Seventy-seven of the 91 loci (84.6%) demonstrated a maximum of one mismatch in 29 samples, suggesting a genotyping error rate of less than 5% for each of these loci, and 63 of the 91 (69.2%) were scored consistently across all replicates. These 63 loci were retained for further analyses. This approach is conservative with regards to locus selection, since studies employing data obtained from genotyping methods such as RAPDs or AFLPs often retain loci with error rates of up to 1–5% (see Bonin et al. 2004; Whitlock et al. 2008). However, while a number of informative and generally reliable loci may have been excluded, the conservative criteria we used responds to many of the concerns regarding the repeatability of RAPD markers (Jones et al. 1997; Pérez et al. 1998; Bagley et al. 2001).

Of the 63 fully repeatable loci, allele frequency estimates identified 34 that were polymorphic in the combined broodstock samples. These included 14 loci that, based on data from a combination of broodstock samples and known first generation hybrids, appear to be diagnostic for either walleye or sauger (Table 3).

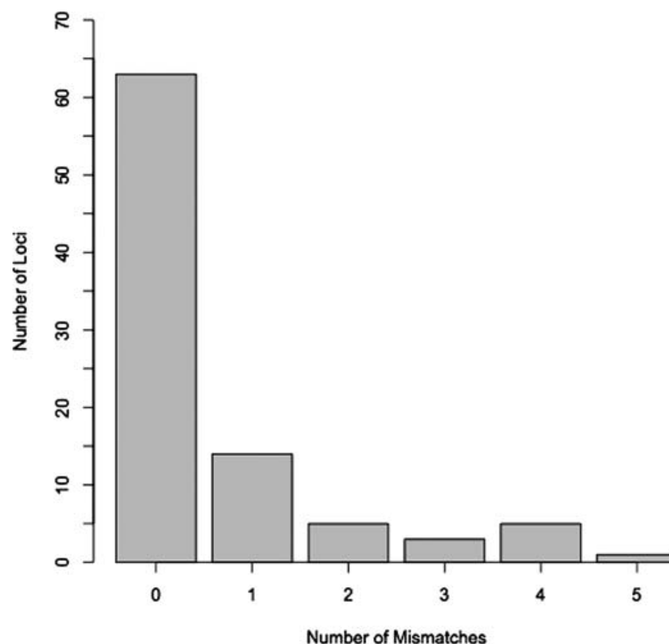

FIGURE 3. Frequencies of loci demonstrating various numbers of mismatches in presence or absence phenotypes after data normalization and scoring of alleles at a 0.1 relative phenotype-calling threshold, as described by Whitlock et al. (2008). Mismatch frequencies for the 91 loci are based on 29 replicated individuals. Of the 91 loci, 63 demonstrated no mismatches across all replicates and were retained for analyses.

To date, diagnostic markers identified in *Sander* spp. have been limited to four allozyme loci (Billington et al. 1996; Van Zee et al. 1996; Fiss et al. 1997; White et al. 2005). Models suggest that the use of four diagnostic loci places the discriminatory power of any test at the extreme low end of the number of markers necessary for a coarse evaluation of parental and hybrid individuals. Achieving distinctions among other classes of hybrid individuals clearly requires larger numbers of loci (Boecklen and Howard 1997). Therefore, identification of 14 diagnostic loci in this study is a valuable improvement, highlighting the utility of the methods we describe.

This set of 14 diagnostic loci was applied to the analysis of 12 age-0 or age-1 saugeye that were collected from Dillon Lake in 2008. Patterns of expression at the diagnostic loci allowed us to determine whether these fish were F_1 hybrid immigrants that had been stocked into waters connected to Dillon Lake, or whether they might be later-generation hybrid offspring of saugeye cohorts that were stocked into Dillon Lake prior to 2006. Ten of the 12 individuals demonstrated the completely additive pattern of allele peaks across all 14 diagnostic loci expected of first-generation hybrids. One of the 12 individuals expressed allele peaks at 13 of the 14 loci. The likelihood that this sample was actually an F_2 individual that was homozygous for the null allele at only one of the 14 diagnostic loci is low (the probability

TABLE 3. Proportion of individuals expressing a band at each of the 34 polymorphic loci. Locus type refers to the conclusion drawn regarding the utility of each locus in addressing questions of hybridization in *Sander* spp. based on allele frequencies in the broodstock inferred in this study. Loci that are polymorphic in at least one of the two parental groups are informative. Loci that are fixed for the presence of an allele in one parental group (based on inferred allele frequencies from parentals and known F_1 hybrids) but show no expression of the band in the other parent are diagnostic and are the most valuable for hybridization studies (indicated by an asterisk).

Primer name	Locus	Proportion of individuals expressing allele			Locus designation
		Walleye ($n = 34$)	Saugeye ($n = 40$)	Sauger ($n = 30$)	
OPM-1	228	0	0.975	1.00	Informative
	664	0.882	0.950	0	Informative
	935*	1.00	1.00	0	Diagnostic
	959*	1.00	1.00	0	Diagnostic
OPM-5	232	0.618	0.350	0	Informative
	425*	1.00	1.00	0	Diagnostic
	568*	1.00	1.00	0	Diagnostic
	888	1.00	0.975	0.933	Informative
OPM-6	321*	0	1.00	1.00	Diagnostic
	642	0.441	0.400	0	Informative
	1033*	0	1.00	1.00	Diagnostic
OPM-10	365	0.971	0.875	0	Informative
	500	0.029	1.00	1.00	Informative
	810	0.029	1.00	1.00	Informative
OPM-11	415	1.00	1.00	0.967	Informative
	784*	0	1.00	1.00	Diagnostic
	975	0	0.00	0.067	Informative
	1163*	0	1.00	1.00	Diagnostic
OPM-12	386	0	0.150	0.400	Informative
	431	0.118	0.050	0	Informative
	457*	1.00	1.00	0	Diagnostic
	497*	0	1.00	1.00	Diagnostic
	803*	0	1.00	1.00	Diagnostic
	821	1.00	0.925	0	Informative
	910*	1.00	1.00	0	Diagnostic
OPM-13	289	1.00	0.950	0	Informative
	461	1.00	1.00	0.067	Informative
	551	1.00	0.850	0	Informative
	569*	0	1.00	1.00	Diagnostic
	612	0.265	0.150	0	Informative
	1075*	0	1.00	1.00	Diagnostic
OPM-14	296	0.147	0.00	0	Informative
	692	0.941	0.650	0	Informative
	728	0.971	1.00	0	Informative

that an F_2 individual is homozygous for the lack of a band peak at any given locus is 0.25). It is much more probable that this individual is an F_1 hybrid that expressed a low frequency absence allele at the locus *OPM-12 497* that was not identified in the sample from the parental broodstock or in known F_1 hybrid samples. This suggests that this locus might not be classified as "diagnostic" if a larger sample of parental types had been examined, although it still remains highly informative for questions of hybridization. Finally, one of the sampled individuals showed band peaks at only seven of the diagnostic loci, indicating that it was not a first-generation hybrid saugeye. However, the specific seven loci were those expressed by walleye broodstock samples. Therefore, this sample is much more likely to be a pure walleye than a later-generation hybrid.

In addition to the 14 loci that we identified as diagnostic, the FRAPD methodology identified 20 additional loci that were

informative, but not diagnostic, with respect to *Sander* spp. hybridization. These included five (*OPM-1 228; OPM-6 1033; OPM-12 821; OPM-13 289; OPM-13 551* in Table 3) that were not included as diagnostic loci, even though the two broodstock samples were fixed for alternative alleles. This was because one or more known F_1 saugeye did not express a peak corresponding to the particular locus.

The set of 20 informative loci were not used in our analysis of the Dillon Lake samples, since applying only the diagnostic loci was sufficient for distinguishing between whether these fish were first-generation hybrids or were some other class of hybrids. However, in many questions of hybridization and introgression, it may be important to try to distinguish among various classes of hybrids (i.e., a first-generation backcross versus the offspring of two first-generation hybrids), and the additional polymorphic informative loci are likely to provide additional power in these cases.

It is possible that additional rare alleles exist in the broodstock populations that were not detected in this study. As a result, some of the loci (i.e., *OPM-12 497*) initially designated as diagnostic may not actually be fully diagnostic. Nevertheless, even if these loci are not fully diagnostic, they remain highly informative in addressing questions of hybridization. Since FRAPD methodology allows relatively rapid and inexpensive screening of additional genetic loci, the potential exists for identifying many more diagnostic loci by applying methods similar to those used in this study to the screening of additional FRAPD primers. There are a very large number of different RAPD primers that are available commercially and could easily be adapted to this methodology, greatly expanding the possibility of identifying a large number of diagnostic markers.

In this study, known first-generation hybrid saugeye were available from hatcheries. These known hybrid saugeye were critical for estimating allele frequencies in broodstock populations, as they permitted the direct estimate of allele frequencies in the parental species that expressed a band, when the other parental species did not express an allele at all. In most questions of hybridization in natural systems, however, it is unlikely that known first-generation hybrids will be available. Without these, the sample sizes required for using dominant markers such as FRAPDs to accurately estimate allele frequencies in the parental populations will be very (often prohibitively) large. As a result, being able to identify diagnostic loci with confidence, as we can do in *Sander* spp., is unlikely with this type of marker. However, a series of genetic analysis software programs such as STRUCTURE (Pritchard et al. 2000) and NewHybrids (Anderson and Thompson 2002) that employ Bayesian statistical methods may be able to account for the uncertainty of not being able to estimate allele frequencies directly. These methods can use information from any polymorphic locus in the sample and therefore provide additional power and versatility when analyzing questions of hybridization with FRAPD data in other systems. Work is ongoing to evaluate the utility of these programs for identifying various classes of species hybrids using FRAPD data, both in *Sander* spp. and in other groups.

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