Billington, N. 1996. Genetic markers and stock Identification. Pages 323–330 *in* **R.** C. Summerfelt, editor. The walleye culture manual. NCRAC Culture Series 101. North Central Regional Aquaculture Center Publications Office, Iowa State University, Ames.

Chapter 14

Genetic Markers and Stock Identification

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Introduction

Genetic differences among geographically separate stocks of walleye have important implications for aquaculture. For example, the natural distribution of walleye ranges from the Mackenzie River in northwestem Canada to northeastern Mississippi (Scott and Crossman 1973), areas with substantial dfferences in climate to which walleye have had to adapt. If aquaculturists wish to raise walleye in outdoor facilities or produce fish that will be sold as fry or fingerlings to stock lakes, ponds or impoundments (currently the major market for walleye aquaculture), broodstock should be chosen from an area with a climate similar to that where they will be cultured. In indoor facilities, temperature and other conditions can be optimized for characteristics of the stock.

The economic viability of walleye aquaculture to food-size fish lies in the development of strains of that are adapted to the environmental conditions and feeding regimens found in commercial aquaculture. Development of a selective breeding program for walleye should begin with a profile of the genetic variation within and among dfferent walleye populations. Population genetic data is needed to recognize unique strains that may be of value for selective breeding programs, to maintain pure strains for aquaculture, and for the protection of native populations. The latter are natural gene banks which are sources of broodstock for future aquacultural manipulation. Within population genetic variation provides a basis for positive gains possible from selective breeding programs as populations with little or no genetic variation have little or no potential. It is important to choose a population with a high level of genetic variation when starting a selective breeding program. Knowledge of the amount of genetic variation, particularly the level of heterozygosity, is thus important in choosing a founder stock for initiating a selective breeding program. In addition, there is the possibility that traits that are to be selected for may be linked to a genetic polymorphism, greatly enhancing the ability to select individuals for use in a broodstock improvement program (Liebowitz et al. 1987).

Genetic markers and stock identification

Programs to ensure the effective management and/or conservation of genetically divergent stocks of a fish species require the identification of such stocks and depend on the availability of genetic markers that can be used to monitor the success of stocking programs. Protein electrophoresis and mitochondrial DNA (mtDNA) analysis have been used to determine genetic markers and stock structure in walleye populations. These techniques are described here and findings on use of protein variation as genetic markers to evaluate stocking strategies will be discussed. In the following review, alleles and loci are given using the nomenclature recommended by Shaklee et al. (1990), although in some cases alleles and loci are described as they were in the original papers.

Protein electrophoresis

Protein molecules tend to have different net electrical charges. When allelic differences occur at a protein-coding locus, the net charge of the protein often changes. Electrophoresis separates mixtures of proteins according to charge, allowing alleles to be distinguished. Generally, electrophoresis involves introducing a solution of soluble proteins into a starch gel, following wh c h the proteins are separated by passing a direct electrical current through the gel. Factors such as the

chemical composition, ionic strength, and pH of the buffer solution can affect the time needed for electrophoresis and the resolution of the protein bands. Most proteins studied are enzymes that are histochemically stained. Details and interpretation of protein electrophoresis and staining methods for fishes can be found in Aebersold et al. (1987), Utter et al. (1987), Buth (1990), and Morizot and Schmidt (1990); information about walleye can be found in Uthe et al. (1966), Clayton et al. (1971), Ward et al. (1989), Billington et al. (1990), Todd (1990), and McInerny et al. (1991). Proteins can also be separated in the buffer-film above a cellulose acetate plate (Hebert and Beaton 1989), and this technique has been shown to be as effective as starch for many polymorphic systems in walleye (McInerny et al. 1991).

The earliest work on protein variation in walleye was conducted by Uthe et al. (1966), who studied muscle myogen proteins in two Canadian populations. They found three phenotypes at the fastest migrating protein zone (locus), with six fish from Great Slave Lake being homozygous for the A pattern, while all three phenotypes (A 10 individuals; B 7 homozygotes; and AB 14 heterozygotes) were found in 31 fish from Lake St. Clair. In addition, other genetically interpretable polymorphisms were found in some minor protein zones. In an expanded survey that included Lake Winnipeg (N = 61), Lake Superior (N = 31), Lake Huron (N = 32), Lake St. Clair (N = 51) and Baptiste Lake in eastern Ontario (**N** = 38). Uthe and Ryder (1970) found the same three main phenotypes. Although there were differences in phenotypic frequencies among lakes, observed frequencies within lakes generally agreed with Hardy-Weinberg expectations. Moreover, they suggested that the geographcal distribution of allelic frequencies among the lakes was consistent with the hypothesis of post-Pleistocene glacial recolonization from two separate refugia, with the ***A** allele being associated with a Mississippian refugium and the **B* allele being associated with an Atlantic refugium. Ward et al. (1989) also examined genetic variation in muscle proteins, identifying four loci, two of which were polymorphic: *PROT-4** (the most anodal locus corresponded to the fast migrating locus of Uthe et al. [1966]), and *PROT-2** (which corresponded to their minor zone polymorphisms). The *PROT-4*100* allele was equivalent to the ***A** allele and the *PROT-4*160* allele was equivalent to the **B* allele of Uthe et al. (1966). Ward et al. (1989) observed geographic distributions of allelic frequencies similar to those observed by Uthe et al. (1966).

The next protein system that was found to be polymorphic in walleye was malate dehydrogenase (MDH) (Enzyme Commission number, E.C. 1.1.1.37). Clayton et al. (1971) found six MDH phenotypes at the second cytosolic (supernatant) MDH locus (*sMDH-B**) in walleye populations from central Canada, the inheritance of which were confirmed in a breeding experiment. They confirmed the presence of three codominant alleles at this locus, which they labeled *C1*, C2 and *C3*, and which correspond to the **70*, **100*, and **140* alleles at the *MDH-3** of Ward et al. (1989). Clayton et al. (1971) found large differences in the frequencies of each allele among four populations: *C1* ranged from zero to 0.556, C2 ranged from 0.143 to 0.641, and C3 ranged from 0.302 to 0.535. They suggested that this variation could be used as a genetic marker in studying the effects of fry stockings. Clayton et al. (1974) extended their previous work by surveying 19 walleye populations from central and western Canada for genetic variation at the *sMDH-B** locus. They found the same three alleles, and observed that their allelic frequencies varied considerably (*C1*, 0.000-0.622; C2, 0.007-0.702; and *C3*, 0.174-0.650). Of particular interest was the fact that the frequency of the *C1* allele exceeded 0.500 in only three populations (Lake Waskesiu, Crean Lake, and Montreal Lake) that were at the headwaters of the Montreal River, in the Prince Albert National Park, Saskatchewan. The 99% confidence intervals for the frequencies of the *C1* alleles from these populations did not overlap with those of any of the other populations, except for the next lake down stream, Egg Lake, which also had a relatively high *C1* allele frequency (0.375). Thus, Clayton et al. (1974) considered the three headwater lakes to have a genetically different walleye stock compared to the other lakes surveyed, with a steep cline in *C1* allele frequencies occurring along the Montreal River.

Ward and Clayton (1975) used variation at the *sMDH-B** locus as a genetic marker to study the effects of walleye fry introductions to West Blue Lake, an isolated lake in Manitoba. As the *C1* allele was not present in West Blue Lake, walleye fry that had been produced by *C1C1* x *C1C1* and *C1C1* x *C1C3* matings were stocked in the lake to evaluate the contribution of the stocked fry to the 1971 and 1972 year-class (Ward and Clayton 1975). Despite high fry mortalities in 1971, stocked fry

contributed 43% to the 1971 year-class, while in 1972 stocked fry appeared to contribute approximately 100% to the 1972 year-class. Schweigert et al. (1977) continued to monitor the contribution of these genetically marked fish to the total catch from 1973 through 1975. They found that the 1971 stocking augmented the total catch between September, 1974 and August, 1975 by only 1.3%, while the 1972 stochng contributed 37.8% of the total catch over the same period. Genetically marked (and also fin-clipped) fingerlings had also been stocked in 1972, and these contributed only 7% to the 1974-1975 total catch, suggesting that fry stockings had made a more successful contribution to the fishery than had stocking fingerlings. Similarly, Mathias et al. (1992) used walleye fry genetically marked by the *sMDH-B*70* allele to determine that stocked walleye contributed about 2.9% to the 1985 year class in Dauphin Lake, Manitoba.

Murphy et al. (1983) also used variation at the *sMDH-B** locus as a genetic marker to evaluate stochng success of walleye in Claytor Lake, Virginia. The lake was originally stocked with walleye from Pennsylvania between 1939-1946 and a self sustaining walleye population became established. In the early 1970's, the walleye population declined and the lake was stocked in 1974, 1975, 1977, and 1979 with fish from Nebraska and Kansas reservoirs that contained walleyes that may have originated in Minnesota. Despite the fact that the stocked fish did not possess unique alleles or phenotypes. Murphy et al. (1983) found that the frequency of the *sMDH-B*-2* allele differed considerably between stocked and unstocked cohorts, which allowed them to estimate the success of supplemental stocking by quantifying changes in cohort allele frequency.

Electrophoretic analysis of MDH and isocitrate dehydrogenase (IDHP) (E.C. 1.1.1.42) was used by Paragamian (1988) to study three Iowa walleye stocks (Mississippi River, Cedar River, and Spirit Lake) to assess the genetic impact of fry stocking in the Cedar River. He found that while phenotypic frequencies of *IDHP-1*1* and *2 alleles did not differ significantly among the three populations, phenotypic frequencies of the *MDH-B*2* and *3 alleles were significantly different in Spirit Lake than in the two river populations, which were similar. Paragamian (1988) used these genetic markers to determine that fry stockings of Spirit Lake walleyes (and their progeny) into Cedar River only contributed about 19% to the Cedar River population. Therefore, he recommended that the stocking program needed reevaluation.

The two alleles at the *IDHP-1** locus have been used as genetic markers in two studies on the comparative stochng success of three size groups of walleye. Jennings and Philipp (1992) produced three groups of walleye each with one of the three possible genotypes (*IDHP-1* 80/80*, **80/100* and **100/100*) to compare the success of stocking walleye as fry, as 50-mm fingerlings, or as 100-mm fingerlings in five northern Illinois impoundments. They quantified the relative success of each stoclung strategy by sampling surviving walleyes by electrofishmg and by assigning individuals to each of the test groups by electrophoretic analysis of tissue from fin clips. They found that survival was highly variable among test groups, lakes, and years. Koppelman et al. (1992), used a similar approach to monitor the stochng success of walleye fry, 38-mm fingerlings, and 102-mm fingerlings in two Missouri impoundments. They found that small fingerlings gave the best survival rates.

While other significantly polymorphc loci (<0.95 frequency of the most common allele) have been found in later walleye electrophoretic studies (Ward et al. 1989; Todd 1990; McInerny et al. 1991), the MDH, IDHP, and muscle myogen or PROT protein systems are particularly useful for electrophoretic studies, as they have been shown to remain stable in storage for up to 10 months in a regular kitchen freezer and for more than three years at ultra-low (-70°C) temperatures (Murphy et al. 1990). The stability of these protein systems enables researchers to conduct electrophoretic analysis months after the samples have been collected.

In most of these studies, protein variation was used as a genetic marker to evaluate stocking strategies. Protein electrophoresis has also been used to discriminate among walleye populations. Wingo (1982) found that a walleye population in the Tombigbee River in northeastern Mississippi exhbited different blood serum protein bands than walleyes from Iowa, New York, and Pennsylvania. Walleye from the Tombigbee River were also unusual in that they were monomorphic at four protein loci that are usually highly polymorphic in walleye (Murphy 1990), providing evidence that they might represent a unique southern stock of walleye. Waltner (1988) found significant differences in allele frequencies at two loci (*sMDH-B** and *PROT-4**) that

allowed discrimination between four walleye populations from South Dakota and a Mississippi River population. She also found highly significant allele frequency differences at *PROT-4** that allowed a glacial lake stock in South Dakota to be discriminated from three upper Missouri River tributary populations. Ward et al. (1989) found that five loci (*ADH**, *IDHP-1**, *sMDH-B**, *PROT-2**, and *PROT-4**) were significantly polymorphic among 15 walleye populations from across the Great Lakes and Manitoba. Four of these loci (*IDHP-1**, *sMDH-B**, *PROT-2**, and *PROT-4**) showed statistically significant allelic frequency differentiation among populations. Ten loci were found to be polymorphic in walleyes sampled from 11 locations in Minnesota, with four loci (*ADH**, *IDHP-1**, *sMDH-B**, and *PROT-4**) exhibiting statistically significant dfferences in allelic frequencies among populations (McInerny et al. 1991). Walleye from Lake Pepin were genetically different from all of the other populations, probably reflecting isolation during the Pleistocene, as Lake Pepin walleye likely originated from the Mississippian refugium whereas the other populations likely originated from a Missourian refugium. Todd (1990) found that the same four loci were sufficiently polymorphic to differentiate walleye populations from western Lake Erie (6 spawning sites) and Lake St. Clair (2 spawning sites). Allelic frequencies differentiating the two walleye stocks were stable between year-classes, sexes, and sample years, demonstrating that fry hatched in a particular area returned there to spawn. Stock-specific homing behavior in Lake St. Clair and western Lake Erie walleye populations was confirmed by additional genetic and physical tagging studes (Todd and Haas 1993).

Mitochondrial DNA analysis

Due to its maternal mode of inheritance, rapid rate of evolution (compared to single copy nuclear DNA), and enhanced susceptibility to genetic bottlenecks (four times that of nuclear DNA), mtDNA has often proved to be a useful genetic marker for stock discrimination in fish populations (Billington and Hebert 1991). Typically, in studies of mtDNA variation, mtDNA is extracted and purified from fresh tissue and digested with restriction endonucleases (enzymes that cut DNA at specific 4-, 5-, or 6-base pair sequences). The resulting fragments are separated according to molecular weight by gel electrophoresis and then visualized with either ethidium bromide staining or by autoradiography (exposing the gel to an X-ray film) after labeling the ends of the DNA fragments with radioactive nucleotides (Ferris and Berg 1987; Billington and Hebert 1988). However, other protocols are available that allow semi-pure mtDNA to be rapidly obtained (Chapman and Brown 1990). If pure mtDNA is not available for analysis on certain specimens, total DNA (mitochondrial and nuclear) can be extracted from frozen or ethanol preserved tissue and then digested with restriction endonucleases. The DNA fragments are separated by electrophoresis, following which the DNA is denatured (to make it single stranded), and transferred and bound to a nylon membrane. Pure mtDNA is radiolabeled, denatured, and used as a hybridization probe to the membrane, where it will only bind to the mtDNA fragments. These fragments are then visualized by autoradiography (Ferris and Berg 1987; Chapman and Brown 1990; Billington and Hebert 1990). Modifications of this technique allow the determination of mtDNA fragment patterns without the need to sacrifice fish (Billington and Hebert 1990).

Variability in mtDNA fragment patterns is interpreted as genetic variation at the nucleotide level. Thus, a nucleotide substitution in the molecule that causes the loss or gain of a nucleotide sequence recognized by a specific restriction enzyme will cause a change in the observed fragment pattern. Composite restriction fragment patterns are determined for a suite of restriction endonucleases that reveal polymorphisms (more than one fragment pattern) and each composite is designated as a mtDNA haplotype. Detailed accounts of mtDNA extraction and analytical protocols can be found elsewhere (Ferris and Berg 1987; Billington and Hebert 1988, 1990, 1991; Chapman and Brown 1990).

An initial study of mtDNA variation in central and eastern Great Lakes walleye populations revealed nine mtDNA haplotypes, which fell into two main groupings (Billington and Hebert 1988). These two groups of haplotypes were thought to represent fish that had recolonized the Great Lakes from two separate Pleistocene glacial refugia, an Atlantic refugium in the east (group A) and the Mississippian refugium (Group B). Ward et al. (1989) expanded their survey westwards to include walleye populations from Lake Superior and northern Manitoba. They found an additional mtDNA haplotype that was present in populations that would have been recolonized with fish from a Missourian refugium (Group C fish). An extensive survey of 847 walleyes from 68 populations from across the whole

range of the species revealed 34 mtDNA haplotypes, of which 21 were only observed in single fish (Billington et al. 1992). Three major walleye haplotypes were identified which had distinct geographic distributions that reflected their origins from the three separate glacial refugia hypothesized by Ward et al. (1989). The other ten haplotypes were locally distributed and could be used for stock identification and to detect past transfers of fish. Analysis of walleyes collected from the Tombigbee River system in northeastern Mississippi revealed that they had a unique divergent mtDNA haplotype (Billington et al. 1992; Billington and Strange 1995), confirming the suggestion by Wingo (1982) and Murphy (1990) that these fish are a genetically unique stock.

The relative resolution of protein electrophoresis and mtDNA analysis in walleye stock identification was examined by Ward et al. (1989), who showed that, consistent with theoretical expectations (Birky et al. 1983), mtDNA data were more effective for stock discrimination than were allozyme data; 37% of the total variation among walleye mtDNA haplotype frequencies could be attributed to inter-population variation, while only 10% of allelic frequency differentiation was linked to inter-populational variation. Ward et al. (1989) also found that mtDNA data provided evidence that the Great Lakes were recolonized by walleye from two glacial refugia, whereas the protein data suggested only a single origin for these populations. This result is a likely consequence of the different modes of inheritance of mtDNA and nuclear DNA markers (Billington and Hebert 1991).

Other uses of genetic markers

Detection of waleye-sauger hybrids

Walleye and sauger hybridize naturally, but rarely. However, saugeye (walleye female x sauger male hybrids) are stocked in a number of states, usually into impoundments as they often perform well in these environments (Humphreys et al. 1984; Leeds 1988). There is also an interest in using saugeye in aquaculture (see chapter on hybrid walleye). Despite the fact that saugeye can have certain aquacultural advantages over walleye, the reciprocal hybrid (sauger female x walleye male) generally does not perform as well (Malison et al. 1990). Hybrids are able to interbreed (backcross) with both walleye and sauger (Hearn 1986), which can result in the introgression of walleye or sauger genomes (introgression is where the genome of one species becomes diluted with that of another species). Introgressive hybridization will likely occur if hybrids escape into waters containing walleye and/or sauger.

As significant genetic differences exist between walleye and sauger for both allozymes and mtDNA (Billington et al. 1990), it is possible to use genetic markers to detect both hybridization and introgression. Billington et al. (1990) found that there were fixed differences at four protein loci between walleye and sauger. A fixed difference is where one species is fixed (100% frequency) for one allele at a locus and the other species is fixed for a different allele at that locus. An F1 hybrid (a first generation cross between the two species) will be heterozygous at each of these diagnostic loci (i.e., a copy of each allele will be found). If two F1 hybrids cross with each other, or if an F1 hybrid backcrosses to either parent species, some of the diagnostic loci will be homozygous and others will be heterozygous. Therefore, these diagnostic loci serve as genetic markers that allow F1 walleye-sauger hybrids and walleye that contain introgressed sauger alleles to be identified. The maternal mode of inheritance of mtDNA allows the female parent of an F1 hybrid to be identified. Thus, if the female parent of a walleye-sauger hybrid was a sauger, the resulting offspring would also have sauger mtDNA, as would any offspring of such hybrid females. Thus, mtDNA analysis can determine introgression of sauger mtDNA into walleye (Billington et al. 1988).

Introgressed individuals containing a mixture of walleye and sauger genomes may not be the most suitable broodstock for developing walleye strains for use in aquaculture. Therefore, walleye broodstock for use in aquaculture should be pure and not possess sauger genes. A careful genetic analysis of potential broodstock should be undertaken to check for introgression of sauger alleles before such fish are used in a selective breeding program for aquaculture. I conducted a survey of allozymes of two walleye populations that were candidates for aquaculture. This survey revealed that fish from one population possessed introgressed sauger alleles and was thus deemed unsuitable for broodstock development.

Broodstock licensing

The development, through selective breedmg, of broodstock for aquaculture is a lengthy process, often taking many generations and may require considerable

financial investment. It is possible to incorporate a genetic marker into such broodstock as a method of identification or as a mark of propriety. **A** simple method of achieving this would be to use females with a rare and distinctive type of mtDNA in the breeding scheme. As such mtDNA markers are selectively neutral, there will be no effect on the selective breedmg process, yet all of the fish produced would possess this distinctive mtDNA. This marker could easily be screened by a nonlethal sampling technique (Billington and Hebert 1990), allowing a particular selected strain to be distinguished from other strains. Markers based on protein variation could also be used (Utter and Seeb 1990) in a similar manner to those used in the walleye stocking studies described earlier. In addition, genetic markers could also provide a method for monitoring the frequency of escapees from fish culture operations in wild stocks (Billington and Hebert 1991).

Acknowledgments

I am grateful to funding from the North Central Region Aquaculture Center that has supported my research on the role of walleye genetics in aquaculture.

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