Identification of Gulf of Mexico Sciaenids by Isoelectric Focusing

Timothy L. King, *Texas Parks and Wildlife Department, Perry R. Bass Marine Fisheries Research Station, HC 2, Box 385, Palacios, TX 77465*

Paul C. Hammerschmidt, *Texas Parks and Wildlife Department, Perry R. Bass Marine Fisheries Research Station, HC 2, Box 385, Palacios, TX 77465*

Eric D. Young, *Texas Parks and Wildlife Department, Perry R. Bass Marine Fisheries Research Station, HC 2, Box 385, Palacios, TX 77465*

Abstract: Sarcoplasmic proteins were isolated from skeletal muscle of 14 species of Gulf of Mexico sciaenids encompassing 11 genera utilizing isoelectric focusing (IEF). Individuals from the 11 genera were distinguishable. However, intrageneric comparisons (*Cynoscion* and *Menticirrhus*) were constrained by similar protein banding among congenerics and required a high resolution pH gradient (pH 4–5) to produce species-specific patterns. A graphical representation of differences in banding patterns among the 14 species was provided by densitometric tracings. Isoelectric focusing provided qualitative evidence of the biochemical relationships among and within the 11 genera surveyed. Although only 2 multi-species genera were surveyed, this study appears to confirm their status as congenerics. Protein profiles generated by IEF appear superior to conventional electrophoretic techniques for the identification of certain sciaenid species. Discrepancies between results of allozyme and isoelectric focusing surveys concerning sciaenid systematics have been identified.

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Sciaenidae are demersal fishes found worldwide in tropical and warm-temperate bays and estuaries of major rivers (Robins and Ray 1986). Those inhabiting the Gulf of Mexico exceed all other families in number of species with approximately 18 species comprising 12 genera (Hoese and Moore 1977). Selected members of this family support valuable recreational fisheries in the Gulf of Mexico. In Texas, 5 sciaenid species account for >80% of recreational sport-boat landings: spotted seatrout (*Cynoscion nebulosus*), Atlantic croaker (*Micropogonias undulatus*), sand

seatrout (*C. arenarius*), red drum (*Sciaenops ocellatus*), and black drum (*Pogonias cromis*) (Green et al. 1992).

Along the Texas gulf coast, recreationally important sciaenids have experienced environmental perturbations and immense fishing pressure which have significantly impacted population sizes (McEachron et al. 1984, Hammerschmidt 1987). Management programs restricting bag, size, and possession limits have been implemented by the Texas Parks and Wildlife Department (TPWD) to check the decline in abundance of red drum, black drum and spotted seatrout (Anonymous 1985, Meador and Green 1986). Further, a ban has been placed on the importation and sale of wild-caught red drum in Texas; only cultured red drum may be imported or sold (State of Texas 1990).

Enforcement of legislatively mandated fish and game regulations in Texas is the responsibility of the Law Enforcement Division of the TPWD. The ability to positively identify cryptic specimens is an essential facet of game-law enforcement. Species identification is complicated when an evidenciary specimen has been altered (e.g., filleted) or only a minute amount of tissue is available (e.g., dried blood). Under litigating circumstances, law enforcement officials must often call upon scientists to provide expert testimony from appropriately applied forensic techniques (Harvey 1990).

Forensic science has recently benefited from robust biochemical identification techniques (Black 1988). Using these techniques, specimens in early stages of decomposition can be identified by electrophoretic separation and identification of species-specific allozymes and peptides (Avise and Van Den Avyle 1984, Lundstrom 1979). Under circumstances of advanced decomposition or low-yield tissues (e.g., scales, hair, blood), polymorphisms in evolutionarily conservative nucleotides of mitochondrial and nuclear DNA molecules can be utilized for species identification (Chapman and Brown 1990, Whitmore et al. 1990).

Isoelectric focusing (IEF) is a high resolution protein separation technique permitting identification of fish species from soluble protein extracts of white muscle (Lundstrom 1979, Whitmore 1986). Parvalbumin proteins isolated by IEF have proven particularly useful to fisheries management and law enforcement agencies for identifying unknown fish samples (Whitmore 1986, 1990; Harvey 1990). Parvalbumins are generally regarded to be stable, species-specific, and to exhibit low levels of electrophoretically detectable intraspecific variability, 3 characteristics which deem these proteins suitable for formation of diagnostic protein profiles (Whitmore 1990). Establishment of a library of protein profiles of economically important sciaenids would serve as "fingerprints" for identification to assist TPWD law enforcement officials with prosecution of fish and game-law violators.

Differences exist among taxonomists as to the number of extant Sciaenidae species and genera; the specific status of some congenerics is questionable (Weinstein and Yerger 1976). The utility of biochemical analyses in resolving systematics problems is widely acknowledged (Avise 1974, Buth 1984). However, the scientific literature is lacking of biochemical investigations into systematic issues of sciaenids. Recently, electrophoresis was applied to the systematic problems that exist among

the western Atlantic seatrouts (*Cynoscion*) (Weinstein and Yerger 1976, Paschall 1986). With the exception of these works, biochemical studies performed have focused on assessment of intraspecific variation (i.e., population structure) (Ramsey and Wakeman 1987, Bohlmeyer and Gold 1991, King and Pate 1992). Little information is available on the taxonomic and evolutionary relationships among many sciaenid species. A survey of general protein patterns using IEF may help elucidate systematics of this important family of fishes.

The objectives of the present study were to: 1) substantiate the utility of IEF for species identification among members of Sciaenidae inhabiting Texas waters; and 2) provide insight into the systematic relationships among members of this family of fishes.

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Methods

Fish were collected from April 1989 to December 1990 by beach seine, gill net, or trawl during routine TPWD sampling (Table 1). Fish were placed on ice or frozen at 0 C and transported to the Perry R. Bass Marine Fisheries Research Station, Palacios, Texas, for processing. Fish were identified to species using dichotomous keys by Hoese and Moore (1977) and Robins and Ray (1986). Skeletal muscle tissue, excised from lateral musculature directly below the first dorsal fin and tissue from the second dorsal fin were removed from thawed specimens. Tissues (diluted 1:1, with deionized water) were homogenized and centrifuged at 10,000 rpm for 10

Table 1. Sciaenidae species incorporated into a library of sarcoplasmic protein profiles generated by isoelectric focusing in pH 4–5 gradient.

Common name	Scientific name	*N*
drum (freshwater)	*Aplodinotus grunniens*	15
silver perch	*Bairdiella chrysoura*	20
sand seatrout	*Cynoscion arenarius*	25
spotted seatrout	*C. nebulosus*	60
silver seatrout	*C. nothus*	20
cubuyu	*Equetus umbrosus*	10
banded drum	*Larimus fasciatus*	10
spot	*Leiostomus xanthurus*	10
southern kingfish	*Menticirrhus americanus*	27
gulf kingfish	*M. littoralis*	20
Atlantic croaker	*Micropogonias undulatus*	30
black drum	*Pogonias cromis*	50
red drum	*Sciaenops ocellatus*	50
star drum	*Stellifer lanceolatus*	10

minutes at 4 C. The resulting supernatant was retained for analysis. Analyses were generally completed within 14 days of collection.

IEF was performed on 0.25-mm polyacrylamide gels consisting of 2 ml of 29.1% (wt/vol) acrylamide and 0.9% N,N'-methylenebisacrylamide, 1.0 ml of ampholytes (0.8 ml pH 4–5 and 0.2 ml pH 3–10 ampholytes; Serva Biochemicals, Westbury, N.Y.), 4.2 ml of deionized water, and 0.8 ml glycerol. Gel solutions were degassed for 5 minutes. After aspiration, 50 μl of 10% TEMED (N,N,N'N'–tetramethylethylenediamine) were added to the gel solution. Polymerization was initiated by addition of 50 μl of 10% ammonium persulfate (APS). The gel solution was then poured onto a GELBOND PAG film (FMC Bioproducts, Rockland, Minn.). Gels were allowed to polymerize for 1 hour before the mold was disassembled and the GELBOND PAG film, with gel attached, was applied to the IEF apparatus.

A LKB 2117 Multiphor II electrofocusing apparatus powered by a LKB 2197 power supply and thermoelectrically cooled by an LKB 2219 Multitemp II thermostatic circulator (Pharmacia LKB Instruments, Gaithersburg, Md. was used to perform IEF. The gel was placed on the cooling platform over a thin layer of Triton X-100 (Pharmacia LKB Instruments, Gaithersburg, Md.) to ensure appropriate thermal conductance. Electrode strips were soaked in 0.5 M acetic acid (anolyte) and 2.0 M ethylenediamine, 0.025 M Arginine, and 0.025 M Lysine (catholyte). Electrode strips were placed parallel to each other at opposite edges of the gel corresponding to the electrodes on the apparatus. IEF was performed at 10 C. Initial current (mA) was adjusted until 200 volts was achieved; final voltage was limited to 1,200 volts. Initial power was set at 4 watts. Gels were prefocused for 20 minutes to ensure establishment of a consistent pH gradient and to rid the gel of excess TEMED and APS. Protein extracts were loaded onto a sample application mask (2 μl capacity; Pharmacia LKB Instruments, Gaithersburg, Md.) and focused 20 minutes after which the mask was removed. The gel was then focused until no decrease in resistance (mA) was observed for 15 minutes (roughly 4 hours).

Isoelectric points (pIs) were assigned to protein bands based on gel pH determinations measured at 10-mm intervals using a surface pH electrode (Pharmacia LKB Instruments, Gaithersburg, Md.) and by comparison with pI values of bands produced by commercial protein markers (Serva Biochemicals, Westbury, N.Y.).

After completion of the run, gels were fixed for 5 minutes in a solution of 4% sulfosalicylic acid and 12.5% trichloroacetic acid. Following fixation, gels were placed in 50 ml of wash solution consisting of 40% methanol and 10% glacial acetic acid for 5 minutes. Protein phenotypes were stained with a 5% Coomassie Blue R-250, 40% methanol, and 10% glacial acetic acid solution for 10 minutes. Gels were destained in wash solution until the background cleared and were allowed to air dry.

Gels were scanned to determine protein migration and intensity (absorbance) utilizing an UltroScan XL Laser Densitometer employing LKB GelScan 2.0 software (Pharmacia LKB Instruments, Gaithersburg, Md.). Species were distinguished based on identification of unique protein bands. Only protein bands occurring in 100% of the samples were assigned pI values.

Results

Sarcoplasmic proteins were isolated from skeletal muscle and dorsal fin tissue of 14 species of Gulf of Mexico sciaenids encompassing 11 genera utilizing IEF (Table 1). Muscle and fin tissue provided identical protein patterns, therefore, only results from muscle are presented. Individuals from all 11 genera were distinguishable. However, intrageneric comparisons (*Cynoscion* and *Menticirrhus*) were constrained by similarities in protein banding among members and required a high resolution pH gradient (pH 4–5) to produce species-specific patterns for all 14 species (Fig. 1). High resolution separation was also necessary to distinguish between banding patterns of silver perch (*Bairdiella chrysoura*) and spotted seatrout. Substantial differences were observed among economically important sciaenids (i.e., red drum, black drum, and spotted seatrout).

A majority of resolvable diagnostic bands were found to have low pI values, whereas little intraspecific variation was detected; rare variants were observed in non-diagnostic proteins. These characteristics suggest surveyed bands were parvalbumin proteins; however, no attempt was made to verify this supposition.

Densitometric tracings provided a discerning graphical representation of differences in banding patterns among the 14 sciaenid species (Fig. 2). Although variation occurred in relative peak heights (absorbance) from sample to sample, the migration distance (from anode) and ratio of absorbance values among putative parvalbumin bands were consistent within a species. Sciaenids surveyed possessed 2–4 parvalbumin bands in the pH 3.58–4.70 range. Eleven sciaenids possessed 1 or 2 bands distinctly more anodal (acidic) than the third band, whereas freshwater drum, banded drum, and star drum appeared to form a unique group exhibiting all parvalbumin bands positioned near the anode. The only predominant morphological characteristic shared by these three species is a well developed second anal spine.

Spotted seatrout was the most distinctive *Cynoscion* species, exhibiting diagnos-

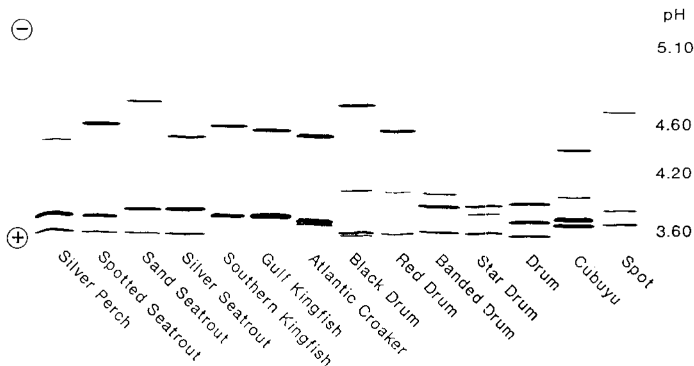

Figure 1. Sarcoplasmic protein patterns from 14 species of Sciaenidae subjected to thin-layer isoelectric focusing in a pH 4–5 gradient.

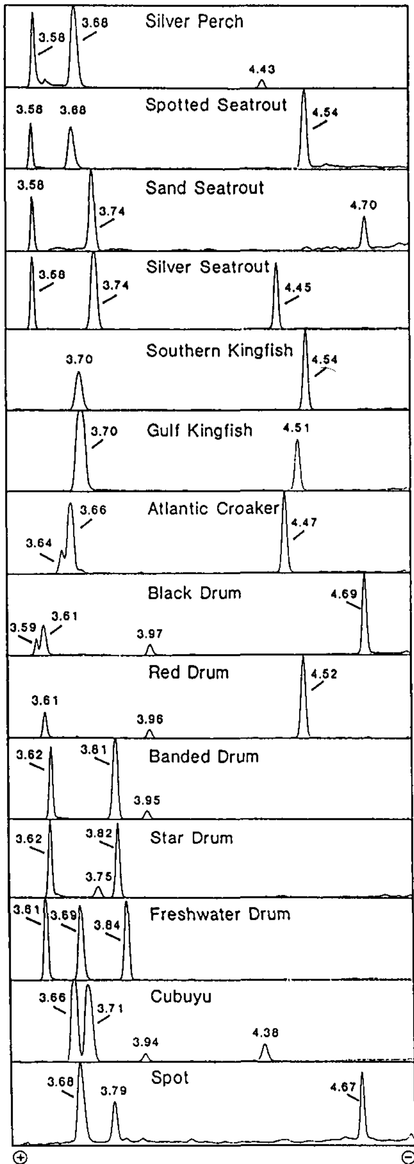

Figure 2. Densitometric tracings and isoelectric points for representative sarcoplasmic protein bands of 14 species of Sciaenidae subjected to isoelectric focusing in a pH 4–5 gradient. Band intensity is indicated by relative peak height. Migration distance is relative to anode (+) electrode strip.

tic differences in 2 protein bands. The most cathodal band in spotted seatrout (pI = 4.54) was intermediate among the corresponding band of sand seatrout (pI = 4.70) and silver seatrout (pI = 4.45); among the seatrouts, the pI = 3.68 band was also diagnostic for the spotted seatrout. The most cathodal parvalbumin bands were the only diagnostic bands observed between the sand seatrout and silver seatrout.

Menticirrhus species were the most difficult to distinguish of all the sciaenids surveyed; only subtle pI differences were observed. The gulf kingfish exhibited a parvalbumin band (pI = 4.51) slightly more anodal than the corresponding band found in southern kingfish (pI = 4.54) (Figs. 1, 2). Efforts to collect the northern kingfish (*M. saxatilis*) were unsuccessful; this species is rarely collected along the Texas gulf coast.

Discussion

High-resolution isoelectric focusing (IEF) of soluble muscle (and fin) proteins permitted accurate identification of the 14 sciaenid species and provided evidence of the biochemical relationships among and within the 11 genera surveyed. Variability in protein phenotypes of putative parvalbumins appeared to correspond well with morphological diversity characteristic among sciaenids. Additionally, parvalbumin proteins appeared to be reflective of taxonomic and evolutionary relationships among members of the Sciaenidae family; congenerics exhibited a high degree of biochemical similarity whereas intergeneric differences were substantial.

Intergeneric electrophoretic differences have been previously identified among sciaenid species (Stanley 1989); however, multiple diagnostic gene loci are required to distinguish similar species. This approach to species identification is generally considered more time intensive, costly, and less discriminating than IEF (Lundstrom 1979, Whitmore 1986, Harvey and Fries 1987). The present study suggests Gulf of Mexico sciaenids can readily be identified by a single IEF run.

IEF was also effective at the more exacting task of intrageneric species identification. The southern and gulf kingfishes were the most arduous species to differentiate morphologically and biochemically. Biochemically they exhibited minor divergence compared to differences observed among other species. This finding suggests speciation (reproductive isolation) may have been relatively recent or that it may be incomplete. Perhaps an extensive allozyme or mitochondrial DNA survey would be appropriate to determine the level of differentiation among the species and to elucidate the systematics of the entire *Menticirrhus* genus.

The distinctive banding pattern exhibited by spotted seatrout supports previous morphological, ecological, and biochemical conclusions concerning *Cynoscion* species. Ginsburg (1929) identified spotted seatrout as the most morphologically distinctive *Cynoscion* species. The predominantly estuarine spotted seatrout are considered ecologically distinct from the other primarily marine *Cynoscion* species (Weinstein and Yerger 1976). Using acrylamide gel electrophoresis (AGE), Weinstein and Yerger (1976) were able to distinguish the three seatrouts using myogen proteins and concluded from general protein banding patterns that spotted seatrout was the most divergent species of the genus.

Comparatively, the protein profiles generated by high resolution IEF provided clearer discrimination of *Cynoscion* species identification than the phenotypes generated by AGE (Weinstein and Yerger 1976; Figs. 4–6, pages 602–3). The banding patterns and their respective densitometric tracings generated for *Cynoscion* by

the 2 techniques were markedly different. Isoelectric focusing produced greater separation and superior resolution of fewer bands than AGE; AGE bands appeared crowded and diffuse.

Cynoscion species were easily discernable from most genera on the basis of parvalbumin proteins. However, a high resolution pH gradient was required to distinguish the silver perch (*Bairdiella chrysoura*) from the spotted seatrout, indicating possible taxonomic similarities. In contrast, Paschall (1986) compared allele frequencies among silver perch, gulf kingfish, southern kingfish, and western Atlantic seatrouts and concluded that silver perch exhibited allelic variation which clustered them more closely to the kingfishes than to either seatrout species.

The reasons for this apparent contradiction are unclear, as is the relationship between taxonomic conclusions drawn from comparisons of allele frequencies of multiple gene loci and myogen protein patterns. How different types of proteins are affected by evolutionary processes such as natural selection and stochastic events is uncertain. The extraordinary resiliency of myogen proteins in the presence of selective forces is broadly accepted (Weinstein and Yerger 1976), whereas the response of allelic variants to the same forces remains controversial (King and Jukes 1969, Wright 1978). The apparent conservatism in structure and amino acid constitution observed in myogen proteins is well documented in fish. Few species have been shown to exhibit detectable polymorphisms (Tsuyuki et al. 1968, Weinstein and Yerger 1976). Consequently, these patterns have proven useful in elucidating taxonomic relationships.

Conclusions

Substantial interspecific differences combined with the lack of intraspecific variation observed in this study suggests that IEF is a robust technique for sciaenid species identification. A library of protein profiles (densitometric tracings) of sciaenid species now exists that can provide law enforcement agencies and fisheries researchers with an accurate means of species identification of unknown sciaenid tissues. This could have a significant impact on future enforcement of fish and game laws since more restrictive bag, possession, and size limits have been placed on the fisheries of red drum, black drum, and spotted seatrout in Texas.

Discrepancies between results of allozyme and isoelectric focusing surveys concerning sciaenid systematics have been identified. Further research should be performed to clarify sciaenid systematics and to elucidate relationships between conclusions drawn from general protein patterns (i.e., parvalbumin) and surveys of multiple gene loci.

Literature Cited

Anonymous. 1985. Saltwater finfish research and management in Texas. Report to the Governor and the 69th Legislature. PWD Report 3000-154. Austin, Texas.

Avise, J.C. 1974. Systematic value of electrophoretic data. Systematic Zool. 23:465–480.

——— and M.J. Van Den Avyle. 1984. Genetic analysis of reproduction of hybrid white bass X striped bass in the Savannah River. Trans. Am. Fish. Soc. 113:563–578.

Black, B. 1988. Evolving legal standards for the admissibility of scientific evidence. Science 239:1508.

Bohlmeyer, D.A. and J.R. Gold. 1991. Genetic studies in marine fishes. II. A protein electrophoretic analysis of population structure in the red drum (*Sciaenops ocellatus*). Mar. Biol. 108:197–206.

Buth, D.G. 1984. The application of isozyme data to systematic problems. Annu. Rev. Ecol. Sys. 15:501–522.

Chapman, R.W. and B.L. Brown. 1990. Mitochondrial DNA isolation methods. Pages 107–129 *in* D. Whitmore, ed. Electrophoretic and isoelectric focusing techniques in fisheries management. CRC Press, Boston, Mass.

Ginsburg, I. 1929. Review of the weakfishes (*Cynoscion*) of the Atlantic and Gulf coasts of the United States, with a description of a new species. Bul. U.S. Bur. Fish. 45:71–85.

Green, L.M., R.P. Campbell, and K.W. Spiller. 1992. Trends in finfish landings, and social and economic characteristics of sport-boat fishermen in Texas marine waters, May 1974–May 1989. Manage. Data Ser. 56. Austin, Texas. (In press).

Hammerschmidt, P.C. 1987. The tide of the red death. Texas Parks and Wildl. Mag. 45:10–15.

Harvey, W.D. 1990. Electrophoretic techniques in forensics and law enforcement. Pages 313–321 *in* D. Whitmore, ed. Electrophoretic and isoelectric focusing techniques in fisheries management. CRC Press, Boston, Mass.

——— and L.T. Fries. 1987. Identification of *Morone* species and congeneric hybrids using isoelectric focusing. Proc. Annu. Conf. Southeast. Assoc. Fish and Wildl. Agencies 41:251–256.

Hoese, H.D. and R.H. Moore. 1977. Fishes of the Gulf of Mexico, Texas, Louisiana, and Adjacent Waters. Texas A&M Univ. Press, College Station, Texas. 327pp.

King, J.L. and T.H. Jukes. 1969. Non-Darwinian evolution. Science 164:788–798.

King, T.L. and H.O. Pate. 1992. Population structure of spotted seatrout inhabiting the Texas Gulf coast: an allozymic perspective. Trans. Am. Fish. Soc. In press.

Lundstrom, R.C. 1979. Fish species identification by thin layer isoelectric focusing. J. Assoc. Off. Anal. Chem. 62:624–629.

McEachron, L.W., G. Saul, J. Cox, C.E. Bryan, and G.C. Matlock. 1984. Winter's arctic weather takes a heavy toll on marine life. Texas Parks and Wildl. Mag. 43:10–13.

Meador, K.L. and A.W. Green. 1986. Effects of a minimum size limit on spotted seatrout recreational harvest. N. Am. J. Fish. Manage. 6:509–518.

Paschall, R.L. 1986. Biochemical systematics of the seatrouts of the western Atlantic, genus *Cynoscion*. M.S. Thesis, Univ. New Orleans, New Orleans, La. 99pp.

Ramsey, P.R. and J.M. Wakeman. 1987. Population structure of *Sciaenops ocellatus* and *Cynoscion nebulosus* (Pisces:Scianidae): biochemical variation, genetic subdivision and dispersal. Copeia 1987:682–695.

Robins, C.R. and G.C. Ray. 1986. A field guide to Atlantic Coast fishes of North America. Houghton Mifflin Co., Boston, Mass. 354pp.

Stanley, J.G. 1989. Stock identification and genetic structure of black drum (*Pogonias cromis*) in the Gulf of Mexico. M.S. Thesis, La. Tech Univ., Ruston. 68pp.

State of Texas. 1990. Texas Parks and Wildlife Laws. West Pub. Co., St. Paul, Minn. 247pp.

Tsuyuki, H., E. Roberts, R.H. Lowes, W. Hadaway, and S.J. Westrheim. 1968. Contribution

of protein electrophoresis to rockfish (Scorpaenidae) systematics. J. Fish. Res. Board Can. 25:2477–2501.

Weinstein, M.P. and R.W. Yerger. 1976. Protein taxonomy of the Gulf of Mexico and Atlantic Ocean seatrouts, genus *Cynoscion*. Fish. Bul. 74:599–607.

Whitmore, D.H. 1986. Identification of sunfish species by muscle protein isoelectric focusing. Comp. Biochem. and Physiol. 84:177–180.

———. 1990. Isoelectric focusing of proteins. Pages 81–105 *in* D. Whitmore, ed. Electrophoretic and isoelectric focusing techniques in fisheries management. CRC Press, Boston, Mass.

———, R. Cotton, and K. Sheridan. 1990. DNA Fingerprinting. Pages 131–141 *in* D. Whitmore, ed. Electrophoretic and isoelectric focusing techniques in fisheries management. CRC Press, Boston, Mass.

Wright, S. 1978. Evolution and the genetics of populations, Vol IV. Variability within and among natural populations. Univ. Chicago Press. Chicago, Ill.