# Population Genetics of *Fundulus heteroclitus* in the Hudson River and North New Jersey Estuaries: Evaluation of Subspecies Boundary and Hybridization with *F. diaphanus*

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## Abstract

The mummichog *Fundulus heteroclitus* is a common estuarine fish, chiefly occupying marshes from Newfoundland to Florida. It consists of two subspecies, very similar by adult morphology, but different genetically, in egg morphology and spawning site preference. *F. heteroclitus heteroclitus* is distributed south from northern New Jersey, while *F. h. macrolepidotus* occurs along the shoreline to the north. According to DNA sequence analyses, these two taxa diverged between 0.5-1.0 million y.a. To reveal the boundary zone between subspecies, mummichogs were sampled in 24 sites between Tuckerton, NJ, New Haven, CT, and Tivoli Bay on the Hudson River. Allozymes were resolved using horizontal starch gel electrophoresis. RFLP-analysis of the PCR-amplified mitochondrial DNA regulatory region was performed to reveal mtDNA haplotype frequencies. Our results indicate that these two subspecies are now isolated by a 120 -150 km area between the Wreck Pond (Sea Girt, NJ) and downriver parts of the Hudson and Hackensack river estuaries, occupied by another subspecies - *Fundulus heteroclitus intermedius* ssp. n. - taxon with clear hybrid origin, but yet genetically distinct from both parent subspecies. *F. h. heteroclitus* and *F. h. intermedius* are now separated by a 5 km long strip of sand beaches between Sea Girt and Belmar, NJ, where a sharp shift in Mdh-A and mtDNA haplotypes frequencies occurs. The heterozygote deficiency for Mdh-A allele in two sites on the Hudson and Hackensack rivers indicates that the highly polymorphic *F. h. intermedius* in NY/NJ Harbor estuaries has become reproductively isolated from *F. h. macrolepidotus*. As a second part of the study, hybridization zones between *F. h. macrolepidotus* and freshwater *F. diaphanus* have been found and characterized in two locations on the Hudson River.

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## Introduction

The mummichog (common killifish) *Fundulus heteroclitus* is an abundant tidal fish, found from southwestern Newfoundland (Leim and Scott, 1966) to northeastern Florida (Relyea, 1983), and very common in the Hudson River tidal estuary from the Battery to Albany, NY. As an abundant generalist predator, it takes an important role in an estuary ecosystem, being a major predator for many important groups in tidal environments: grass shrimp *Palaeomonetes spp.*, gammarids, snails and, in fresh water, chironomids and other benthic species. On the other hand, it serves as a prey for the larger fish such as white perch *Morone americana* (Miller, 1963), and for blue crab *Callinectes sapidus* (Kneib, 1982). Spawning takes place from May until August, with a peak reached every two weeks (driven by spring tides). Fish reach maturity on the second year, and some survive over 3 years, attaining a maximum length of about 140 mm. The banded killifish *Fundulus diaphanus* inhabits the freshwater Hudson estuary and it widely overlaps with *F. heteroclitus*. Both species are valuable bait fish, obtained by minnow traps, or seine.

### Variation in mummichog morphology and problems in subspecies recognition.

At the beginning of the century, variations in adult morphology led to recognition of five subspecies of *Fundulus heteroclitus*: *grandis*, *bermudae*, *fonticola*, *macrolepidotus*, and *heteroclitus* but it was mentioned, that "more thorough study is required before the validity of the several subspecies of *Fundulus heteroclitus* can be regarded as well established" (Hubbs, 1926). Later the first two subspecies had been upgraded to the species level (Broun, 1957; Relyea, 1983; Able and Felley, 1988; Duggins et al., 1990) and the third, *fonticola*, rejected as not valid. Relyea (1983) found some variation between *F. h. heteroclitus* and *F. h. macrolepidotus* in gill raker number and female anal sheath length, but decided not to fragment *F. heteroclitus* into subspecies. Based on

concordance in a large set of meristic, morphological, and genetic data, Able and Felley (1986) suggest, that "These morphs should be treated as a separate taxa, with *F. h. heteroclitus* occurring along the east coast from New Jersey south to Florida, including lower Chesapeake and Delaware bays and *F. h. macrolepidotus* distributed from Connecticut north to Newfoundland with disjunct populations in upper Chesapeake and Delaware bays." Lack of morphological diagnostic characters make field recognition of the subspecies very difficult, if possible.

Major important differences may be summarized as follows:

(1) Morphometric characters: northern specimens have fewer predorsal scales, lateral line scales, and gill rakers, and longer and shallower heads and larger eyes than southern specimens (Able and Felley, 1986).

(2) Egg size and morphology: northern populations are characterized by smaller eggs, a low number of oil droplets, and relatively few, but long, thick filaments on the eggs (Able and Felley 1986, Marteinsdottir and Able, 1988).

(3) Spawning site preference: In southern populations, females usually deposit the eggs on *Spartina alterniflora* or between the empty shells of the ribbed mussel, *Geukensia demissa*, while the females from northern sites prefer to deposit their eggs in algal mats or directly in sand (Taylor, 1986). Also, the northern population prefers higher salinity for spawning sites than southern (Able and Palmer, 1988).



**Genetic variation along the Atlantic coast.**

The mummichog was one of the first fish species for which genetic polymorphism was reported (Griffith, 1968; Holmes and Whitt, 1970; Whitt, 1970). Mitton and Koehn (1974) found that greater than 50 % of 25 loci scored were polymorphic with an average heterozygosity of 18.5% per locus per individual. Powers and coworkers extended their study and found 16 out of 45 loci to be polymorphic (Powers et al., 1986; Ropson et al., 1990). Examination of the geographical distribution uncovered significant directional changes in gene frequency (i.e., clines) and a degree of heterozygosity with latitude at some loci (Powers et al., 1986).

Lactate dehydrogenase-B **(Ldh-B)** has two predominant alleles: a fast allele (a) fixed at 1.00 at Savannah, GA (latitude 32.08N), whose frequency slowly decreases northward to 0.627 at Point Pleasant, NJ (40.09N). Then, within a less then degree latitude it drops down to 0.25 (Stony Brook, NY (40.93N)) , and keeps decreasing slowly to 0.00 at Shediac, NB, Canada (46.25N) (Powers and Place, 1978; Ropson et al., 1990).

Malate dehydrogenase-A (**Mdh-A**) has a significantly sharper cline at the same area, being almost fixed to the north and south from NY Bay for (a) and (b) alleles. Between Point Pleasant and Stony Brook there is a shift from 0.020 to 0.952 for the fast allele (Powers and Place, 1978).

Glucosephosphate isomerase-B (**Gpi-B**), is fixed only at one, the north end of the distribution. Four alleles are known at this locus, two (b and c) being common and two other (a and d) being present in less than 5% of the individuals scored (Place and Powers, 1978). The (c) allele frequency reaches 1.00 at Bar Harbor, ME (44.43N), but few individuals with (b) occur northward (Ropson et al., 1990); it decreases to the south to 0.895 at Stony Brook. From Point Pleasant to the southern edge of the species distribution it fluctuates between 0.654 and 0.234 without clear clinal pattern (Powers and Place, 1978).

Mannosephosphate isomerase-A (**Mpi-A**) may be considered variable only within the north

subspecies populations. It has four alleles (a,b,c,d), two of which are rare (less then 5 %(a) and 8 %(c)). Being about 10% within all *F. h. heteroclitus* populations, allele (d) has significant variation (0.172 to 1.00) in *F. h. macrolepidotus* north from Sandy Hook. The most sharp shift in allele frequency (0.286-0.890) occurs between populations on different sides of Cape Cod (Ropson et al., 1990).

Hexose-6-phosphate dehydrogenase-A (**H-6-pdh-A**), like the previous enzyme, has a major shift in allele (c) gene frequency from the S to N side of Cape Cod. (from 0.415 to 0.857), and further north it becomes fixed at 1.00. In *F. h. heteroclitus* it fluctuates smoothly between 0.06 and 0.294 without any clinal patterns.

Aspartate amino transferase-A (**Aat-A**) has substantial variation only in the middle of *F.h.macrolepidotus* range - at Wiscasset, ME (frequency of rare allele (b)=0.358) and Salisbury, MA (0.185). In other populations this allele never exceeds 2%. The second locus, Aat-B, shows some degree of polymorphism in all populations studied (Ropson et al., 1990).

Esterase-B (**Est-B**), Isocitrate dehydrogenase -A (**Idh-A**), Idh-B, Fumarase-A (**Fum-A**), and Phosphoglucomutase-B (**Pgm-B**), and **Pgm-A** have some degree of polymorphism in all populations studied, except the most northern, where the most common allele became fixed at 1.00. Only Acid phosphatase-A (**Ap-A**) and Esterase-C (**Est-C**) remain at the same level of polymorphism through the all populations studied (Powers and Place, 1978; Powers et al., 1986; Brown et al., 1988; Ropson et al., 1990).

Large phylogenetic distance has been revealed between the two subspecies when mitochondrial DNA restriction fragment length polymorphism (RFLP) technique has been applied. The specimens from Wiscasset, ME, and 11 out of 12 specimens from Vince Lombardi rest stop (**VL**) (Ridgefield Borough, NJ) were found to posses a "north" haplotype, while all specimens from Sapelo Island, GA, Stony Harbor NJ, and 1 from **VL** were found to have the "south" haplotype. Even though



southern populations were polymorphic, the distance between the two subspecies was found about 1% of all mtDNA sequence, and the time of divergence was estimated as 1 my (Gonzales-Villasenor and Powers, 1990).

Molecular phylogenies using mitochondrial DNA (cytochrome b gene) and nuclear alleles of the lactate dehydrogenase B locus have been studied (Bernardi et al., 1993). Both mtDNA and lactate dehydrogenase alleles showed a clear separation between the northern individuals (from Nova Scotia and Maine) and the southern ones (from Georgia and Florida), with estimated time of genetic isolation 0.5-1.0 my.

**Genetic variation in Chesapeake and Delaware Bays.**

Chesapeake Bay and Delaware Bay are large tidal estuaries, extended from inlets toward the north, almost parallel to the Atlantic coast. Distribution of chorion filament density and oil droplets in eggs and adult morphometry were analyzed by Morin and Able (1983) and Able and Felley (1986), while allozyme frequencies and mtDNA were studied by Powers and coworkers (Powers et al., 1986, Smith et al., 1992; Powers et al., 1993). All studies revealed the existence of *F. h. heteroclitus* in lower bays, and *F. h. macrolepidotus* in upper bay areas, with at least four subspecies boundaries - in Delaware Bay, in upper Chesapeake Bay, and in lower parts of the Potomac and James Rivers - two major Chesapeake Bay tributaries. The most steep and concordant clines, which may be used as diagnostic characters, are mtDNA haplotypes, Mdh-A, and chorion filament density. The frequency of north mtDNA genotype increases tremendously from 0.04% to 100% within 19 miles up the James River, with the same tendency in other tributaries of the Chesapeake Bay. Distribution of allozyme frequencies indicates an asymmetric hybridization between two taxa - dispersion of "north" alleles south from the boundaries is much wider then "south" alleles to the north, (drawn from the references mentioned above).

***Fundulus diaphanus* in sympatry with *F. heteroclitus*: niche partitioning and hybridization.**

The ranges of *F. heteroclitus* and *F. diaphanus* are widely overlapping, and they were reported as coinhabitants in the Hudson River south to Poughkeepsie, NY (Perlmutter et al., 1967), as well as in some NJ estuaries (Hastings and Good, 1977 - Delaware River tributaries; Tatham et al., 1984 - Barnegat Bay). Although *F. heteroclitus* is considered to be euryhaline, and *F. diaphanus* essentially fresh water, growth rate and fecundity of banded killifish were greater in brackish water then in typical fresh water environment (Fritz and Garside, 1975) and this species has been shown to be under less physiological stress when kept in brackish water (Garside and Jordan, 1968). However, when the salinity preference of this brackish population was tested experimentally, they had a strong preference for fresh water (Fritz and Garside, 1974). The avoidance of brackish water by banded killifish may be explained if we assume, that *F. diaphanus* has been ousted from there by a more adapted niche competitor, most probably by *F.heteroclitus*. On the other hand, mummichogs are rarely found in nontidal freshwater sites, and may be driven from there by freshwater congeners. The interaction at the end of tidal influence in large freshwater estuaries such as the Hudson River may be of interest.

Although *F. heteroclitus* and *F. diaphanus* belong to two subgenera, these two species are hybridizing under laboratory condition and $F_1$ hybrids are viable (R. Dawley, personal communication). Cases of natural hybridization also have been reported. Hubbs and co-workers described one female hybrid from a freshwater lake on Prince Edward Island, in the Gulf of St. Lawrence, Canada (Hubbs et al., 1943). All-female hybrids are known from two estuaries in Nova Scotia, Canada (Fritz and Garside, 1975). Allozyme data show that these hybrids are genetically identical and are gynogenetic clones (Dawley 1992). Occurrence of natural hybrids in the New Haven, CT vicinity was reported, and study of esterase polymorphisms confirmed the hybrid origin



of these fish (Griffith, 1968). Chen and Ruddle (1970) confirmed hybridization by karyotypic analysis, and noted that all specimens caught were females.

Occurrence of hybrids between *F. diaphanus* and *heteroclitus* has never been reported in the Hudson River. Because hybrids are very similar to *F. heteroclitus* females in morphology and therefore could be overlooked, some mummichog populations have been screened for the presence of *F. diaphanus* diagnostic loci to reveal hybridization between these two species.

The purpose of our study was to locate the dividing line, and to determine the degree of intergradation between *Fundulus h. heteroclitus* and *F. h. macrolepidotus*, and to test the possibility that *F. heteroclitus* may hybridize with sympatric populations of *F.diaphanus* in the Hudson River.

## Methods

**Sample sites.**

According to the objectives of our study, we tried to collect fish evenly from the Battery upstream throughout the Hudson River estuary. Seven locations were sampled (**Fig. 1**).

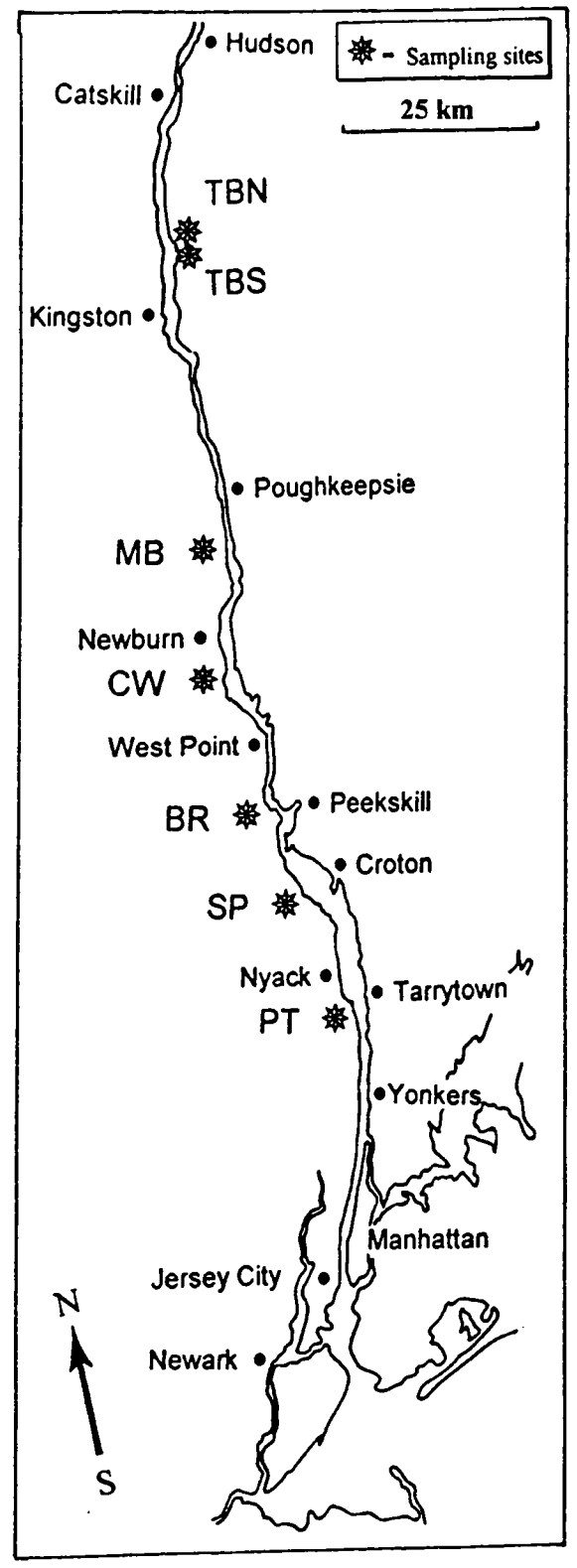
**I.** Staten Island, NY (**PG**). Due to the complete absence of shallow vegetated marshes in the low part of Hudson River, a small creek on the Proctor & Gamble Co. property at Northeast coast of Staten Island was chosen. Although this creek belongs to the south part of Newark Bay rather then the Hudson River, it obviously represents the lower Hudson River mummichog population.

**II.** Piermont, NY (**PT**). Tallman Mountain State Park include a large area of unimproved Hudson River waterfront. Sampling site was on the right bank of Sparkill Creek in this park, off the bridge on Piermont Avenue.

**III.** Stony Point, NY (**SP**) Fish were caught on the right side of the Minnisceongo Creek just before it flows into the Hudson River (near the bridge on the Grassy Point Rd.). No mummichogs were caught in a site 1 km upstream the Minnisceongo Creek.



**Figure 1.** Sample sites on the Hudson River.



**IV.** Iona Island **(BR).** Doodletown Bight of the Iona Island Bird Sanctuary off the road 9W. Mummichogs are abundant in the small creeks, filled during the high tide.

**V.** Cornwall-on-Hudson, NY. **(CW).** Wide shallow marshes where the Moodna Creek approaches the Hudson River. Sampling site is on the Shore Rd. near the Conrail bridge.

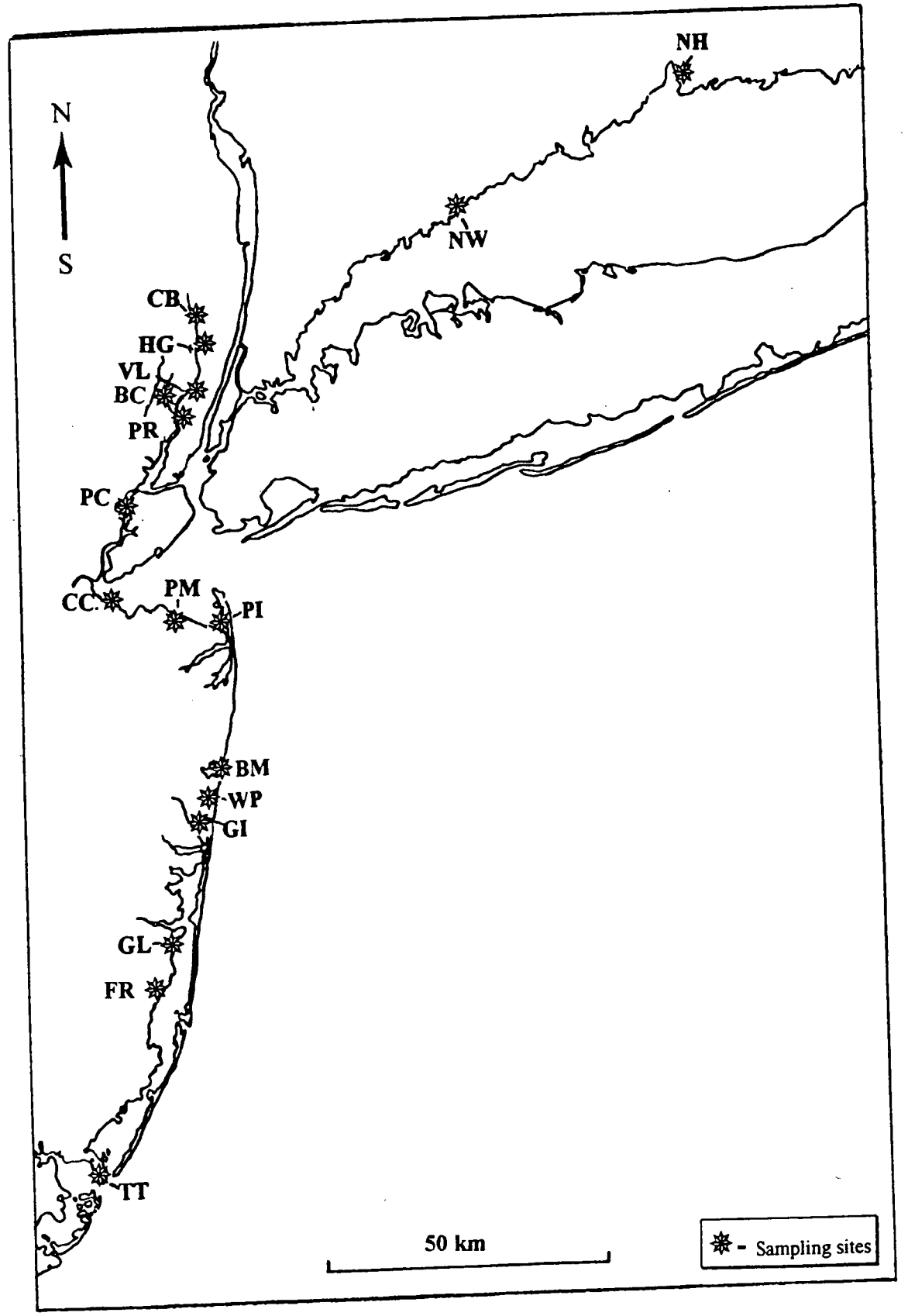
**VI.** Marlboro, NY. Two sites were chosen on Lattintown Creek. One, **(MB1)** is on the right side of the creek, 30 m from the Hudson River, and the second, **(MB2)** is 1 km upstream the creek on the left side, near the end of the tidal influence. Both sites have a soft sandy bottom and vegetation.

**VII.** Tivoli Bay. Two sites with different environments were chosen. One **(TBS)** is near the Bard College Field Station, where Saw Kill goes into South Tivoli Bay. The site is characterized by rocky bottom and abundant water chestnut (*Trapa natans*). Influence of Saw Kill inlet should lead to a relatively high level of dissolved oxygen. Second site **(TBN)** is on North Tivoli Bay near Stony Creek and represents a "classical" tidal marsh creek with deep sulfur-smelling mud.

When mtDNA analysis revealed the north/south haplotype boundary lies south of Raritan Bay, fish were collected at five locations along the New Jersey shore, in addition to the Hudson River sites.



**Figure 2**. Sample sites in New Jersey (including the Hackensack River) and Connecticut.



**VIII.** Shark River, Belmar, NJ **(BM).** Fish were collected at marshes on the west side of Shark River Island, and on the ocean side of the rock jetties, where mummichogs seem to be abundant throughout the summer.

**IX.** Wreck Pond, Sea Girt, NJ **(WP).** Fish were collected in Wreck Pond, which is partially regulated by a dam. In the low portion of this pond salinity fluctuates from 10 to 25 ppt, according to the tide. Mud flats on the south side of the pond have abundant mummichogs mixed with *Fundulus diaphanus*. The upper part of this pond, and Mill Pond (1 km upstream of the Wreck Pond Brook), are essentially fresh water, and only perch and sunfishes were found.

**X.** Manasquan River, Point Pleasant Beach, NJ **(GI).** Fish were collected on the Gull Island, near the railroad bridge.

**XI.** Good Luck Point, NJ **(GL).** The small tidal pool where fish were collected is about $25m^2$, and connects with Barnegat Bay by a very narrow, long (about 200 m) and shallow canal.

**XII.** Forked River, Ocean County, NJ **(FR).** Fish were collected at Forked River at the end of Lacey Rd. Forked River is part of a water intake system of Oyster Creek Nuclear Generating Station, and is a site with ambient water temperatures (not affected by thermal pollution).

**XIII.** Tuckerton, NJ **(TT).** Several tidal ponds and creeks were sampled in the vicinity of Rutgers University Marine Field Station.



Fish were collected by minnow traps baited with bread or canned cat food, by 7 m long minnow seine, or both. Fish were packed in plastic ziplock bags and frozen on a block of dry ice at site, or transported alive with a battery-operated air pump and placed in aquarium tanks until used. Live fish were anesthetized by placing in an ice bath for 5 min., and sacrificed by cutting the spinal cord behind the skull. Specimens were visually identified with respect to species (*F. diaphanus* vs. *F. heteroclitus*), the sex of individuals was determined, and standard length and dorsal fin rays count were recorded. Eyeball, liver, and skeletal muscle from the tail region were removed for genetic analyses and placed in labeled 1.5 ml. Eppendorf tubes. The individually labeled carcasses were preserved in 70% ethanol and stored for further morphometric analyses. If tissue samples were not used immediately after dissection, tubes were frozen at -80°C.

**Starch gel electrophoresis.**

Protein electrophoresis was conducted using standard techniques (Murphy et al., 1990). Eye and liver samples are crudely homogenized in a Tris-HCl grinding buffer (pH 8.0), spun in a centrifuge (10,000 g for 5 minutes), and the supernatant applied into preformed wells in 12% starch gels. Starch gel buffer systems are described in Mulvey and Vrijenhoek (1981). To obtain the best resolution for Mdh-A allozyme, gels with applied eyeball extract were run for 15-20 hrs in the cold room at +4C and 50 mA per gel using Tris-Citric acid-EDTA buffer, adjusted to pH 7.0. Usually this run allows one to score Mdh-A, Ldh-B , Pgi-B, and Pgm-A loci. To score the Mpi-A, 6Pgdh-A, Pgm-B, Idh-A, and Idh-B, liver extracts were resolved using the same buffer, but for a shorter time. Genetic interpretation of electrophoretic patterns is based on previous genetic analyses (Powers et al. 1986). Population genetic analyses was performed on the BIOSYS-I program (Swofford and Selander, 1981).

**Mitochondrial DNA analysis.**

Mitochondrial DNA was extracted with nuclear DNA from muscle tissue. Tissues were crudely homogenized in CTAB extraction buffer (Karl and Avise, 1992). Regions of the regulatory region ("D-loop")of mtDNA molecule were amplified with two primers: H-TDKD (5'-CCTGAAGTAGGAACCAGATG-3' Meyer at al., 1990) and L-ProF (A. Meyer, unpublished). PCR-amplified products were cut with restriction enzymes directed at the diagnostic sites. Fourteen restriction enzymes were tested to reveal diagnostic restriction fragment length polymorphism (RFLP) between *F. h. heteroclitus* and *F. h. macrolepidotus*. Six restriction enzymes showed distinct patterns, and two (Dde-I and Scr F-I) were chosen for routine analyses. Haplotypes were assessed as "north" (n) or "south" (s), when digestion patterns by Dde I and Scr F-I were concordant (only one specimen out of 50 studied showed the pattern, interpreted as "north" for one, and as a "south" for another restrictase product).

**Evaluation of "hybrid index" between *F.heteroclitus* and *F. diaphanus*.**

In two populations, (**MB2** and **TBS**), where intergradation between these two species had been found, four diagnostic loci, which are fixed in both species, were used for calculating the hybrid index. Ldh-E, Ldh-B, and Pgi-B were scored, and index 0 was applied to individuals which had all four alleles "*diaphanus*-type". Each "*heteroclitus*-type" allele possessed by a specimen added 1 point to its hybrid index, so four "*heteroclitus*-type" homozygotes (pure *F.heteroclitus*) were assessed as index 6.



## Results

***Fundulus heteroclitus.***

The gene frequency distribution within the Hudson River resembles that previously found in the Hackensack River. Being very polymorphic in Raritan Bay, mummichog populations upstream became less polymorphic, and even homozygous for some alleles. **Table 1** combines allele frequencies for Mdh-A, Ldh-B, Pgi-A, and Pgm-A in the Hackensack River and along the Connecticut shore, our recent results from the Hudson River and north New Jersey sites, and mtDNA haplotype frequencies for the sites studied.

The most steep cline occurs between Wreck Pond and Shark River estuaries, where both mtDNA and Mdh-A allelic frequencies shift significantly within 5 km and clearly indicates the recent subspecies' boundary. Being absent at Tuckerton, the northern pattern of mitochondrial DNA haplotype (mtDNA(n)) appears in fish caught in the Good Luck Point in Barnegat Bay (20%), and slowly increase over the next 23 km to 50% at Manasquan River (at Gull Island), and remains at approximately the same level at Wreck Pond (47.3%). Within 5 km toward north from Wreck Pond, mtDNA(n) is found in 87% of the mummichogs analyzed from Shark River (**BM**), and completely replaces the southern haplotypes in all populations to the North from the Shark River **Fig. 3a.**

The genetic difference between populations separated by a short strip of sand beaches from Wreck Pond to Shark River is also manifested by one nuclear DNA marker - Mdh-A gene. All Atlantic shore populations from Tuckerton to the Manasquan River are fixed or nearly fixed in Mdh-Ab , making this allele a reliable marker for *F.heteroclitus heteroclitus*. The frequency of another allele of this locus, Mdh-Aa , increases from 6% in Manasquan River (**GI**) to 22% at Wreck Pond (**WP**). In Shark River at Belmar (65.7%) to the North it remains the major allele **(Figs. 3a and 3b).** Northward from Belmar to the southern parts of the Hudson and Hackensack

river estuaries Mdh-Aa remains at an average frequency of 60%, presented as a plateau on the plot frequency vs. distance. After 269 km on the Hudson River (Iona Island, **BR**) **(Fig. 3b),** and even more noticeable after 210 km on the Hackensack River (Vince Lombardi, **VL**) **(fig. 3a),** the second cline occurs, and Mdh-A frequency approaches fixation (88% at **HG** and 97% at **MB**).

Three other loci, analyzed throughout the study area do not show significant shift in allele frequency concordant with Mdh-A and mtDNA clines and are not suitable to define the subspecies boundary. However, all these loci reveal a tremendous decrease of polymorphism toward the most upstream sites **(Table 1).**

Each locus was tested by $X^2$-analysis for concordance to Hardy-Weinberg equilibrium within each population. No significant deviation was found for Ldh-B, PGM-A, and Gpi-A loci. However, high fixation indices and significant deficiency of heterozygotes were found for Mdh-A at two sites - Iona Island (**BR**) on the Hudson River and Vince Lombardi Rest Stop (**VL**) on the Hackensack River. **(Table 2)** - the same sites where second cline of gene frequency occurs.

To find out the origin of the discrepancy to H-W equilibrium at these sites, the sample at **VL** was divided into two subsamples: small (<40 mm) mummichogs, which represent young-of-the- . year cohort, and big ones (40 mm), which are overwintered one to three year



| Site | km | mtDNA (N) | mtDNA N | mtDNA S | mtDNA SE | Mdh-A (N) | Mdh-A A | Mdh-A B | Mdh-A C | Mdh-A SE(A) | Ldh-B (N) | Ldh-B A | Ldh-B B | Ldh-B C | Ldh-B SE(A) | Pgm-A (N) | Pgm-A A | Pgm-A B | Pgm-A C | Pgm-A SE(B) | Pgi-A (N) | Pgi-A A | Pgi-A B | Pgi-A C | Pgi-A D | Pgi-A SE(B) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **New Jersey - Raritan Bays** | | | | | | | | | | | | | | | | | | | | | | | | | |
| TT | 0 | 5 | 0.000 | 1.000 | 0.000 | 65 | 0.000 | 1.000 | 0.000 | 0.000 | 65 | 0.569 | 0.431 | 0.000 | 0.043 | 65 | 0.008 | 0.800 | 0.192 | 0.035 | 65 | 0.000 | 0.415 | 0.585 | 0.000 | 0.043 |
| FR | 45 | - | - | - | - | 29 | 0.000 | 1.000 | 0.000 | 0.000 | 29 | 0.655 | 0.328 | 0.017 | 0.062 | 29 | 0.000 | 0.793 | 0.207 | 0.053 | 29 | 0.000 | 0.328 | 0.672 | 0.000 | 0.062 |
| GL | 55 | 5 | 0.200 | 0.400 | 0.179 | 237 | 0.019 | 0.979 | 0.002 | 0.006 | 213 | 0.667 | 0.333 | 0.000 | 0.023 | 60 | 0.008 | 0.817 | 0.175 | 0.035 | 181 | 0.006 | 0.406 | 0.583 | 0.006 | 0.026 |
| GI | 78 | 20 | 0.500 | 0.500 | 0.112 | 42 | 0.060 | 0.917 | 0.024 | 0.026 | 42 | 0.452 | 0.548 | 0.000 | 0.054 | 10 | 0.000 | 0.700 | 0.300 | 0.102 | 42 | 0.000 | 0.262 | 0.738 | 0.000 | 0.048 |
| WP | 83 | 19 | 0.473 | 0.527 | 0.115 | 25 | 0.220 | 0.780 | 0.000 | 0.059 | 25 | 0.680 | 0.320 | 0.000 | 0.066 | 10 | 0.000 | 0.800 | 0.200 | 0.089 | 25 | 0.000 | 0.400 | 0.600 | 0.000 | 0.069 |
| BM | 88 | 29 | 0.870 | 0.130 | 0.062 | 89 | 0.567 | 0.433 | 0.000 | 0.037 | 87 | 0.454 | 0.546 | 0.000 | 0.038 | 74 | 0.007 | 0.872 | 0.122 | 0.027 | 89 | 0.000 | 0.292 | 0.708 | 0.000 | 0.034 |
| PI | 135 | - | - | - | - | 25 | 0.540 | 0.460 | 0.000 | 0.070 | 25 | 0.540 | 0.460 | 0.000 | 0.070 | 20 | 0.050 | 0.850 | 0.100 | 0.056 | 20 | 0.000 | 0.200 | 0.800 | 0.000 | 0.063 |
| PM | 145 | - | - | - | - | 20 | 0.600 | 0.400 | 0.000 | 0.077 | 20 | 0.350 | 0.650 | 0.000 | 0.079 | 20 | 0.050 | 0.850 | 0.100 | 0.055 | 15 | 0.000 | 0.133 | 0.867 | 0.000 | 0.062 |
| CC | 164 | - | - | - | - | 15 | 0.500 | 0.500 | 0.000 | 0.091 | 15 | 0.333 | 0.667 | 0.000 | 0.086 | 15 | 0.000 | 0.900 | 0.100 | 0.055 | 15 | 0.000 | 0.250 | 0.750 | 0.000 | 0.054 |
| PC | 182 | 5 | 1.000 | 0.000 | 0.000 | 32 | 0.672 | 0.328 | 0.000 | 0.059 | 32 | 0.516 | 0.484 | 0.000 | 0.062 | 32 | 0.000 | 0.922 | 0.078 | 0.034 | 32 | 0.000 | 0.250 | 0.750 | 0.000 | - |
| **Connecticut** | | | | | | | | | | | | | | | | | | | | | | | | | |
| NW | 269 | - | - | - | - | 29 | 0.897 | 0.103 | 0.000 | 0.040 | 29 | 0.310 | 0.690 | 0.000 | 0.061 | 19 | 0.000 | 1.000 | 0.000 | 0.000 | 29 | 0.000 | 0.155 | 0.845 | 0.000 | 0.048 |
| NH | 318 | 5 | 1.000 | 0.000 | 0.000 | 45 | 0.967 | 0.033 | 0.000 | 0.019 | 45 | 0.156 | 0.844 | 0.000 | 0.038 | 44 | 0.000 | 0.989 | 0.011 | 0.011 | 44 | 0.000 | 0.102 | 0.886 | 0.011 | 0.032 |
| **Hudson River** | | | | | | | | | | | | | | | | | | | | | | | | | |
| PL | 236 | - | - | - | - | 20 | 0.650 | 0.350 | 0.000 | 0.075 | 20 | 0.450 | 0.550 | 0.000 | 0.079 | 20 | 0.000 | 0.975 | 0.025 | 0.025 | 20 | 0.000 | 0.075 | 0.925 | 0.000 | 0.042 |
| SP | 257 | - | - | - | - | 26 | 0.673 | 0.327 | 0.000 | 0.065 | 26 | 0.269 | 0.731 | 0.000 | 0.061 | 16 | 0.000 | 0.969 | 0.031 | 0.031 | 16 | 0.000 | 0.063 | 0.938 | 0.000 | 0.043 |
| BR | 269 | 9 | 1.000 | 0.000 | 0.000 | 28 | 0.696 | 0.304 | 0.000 | 0.061 | 28 | 0.321 | 0.679 | 0.000 | 0.062 | 10 | 0.000 | 1.000 | 0.000 | 0.000 | 14 | 0.000 | 0.071 | 0.929 | 0.000 | 0.049 |
| CW | 287 | 8 | 0.625 | 0.375 | 0.171 | 21 | 0.857 | 0.143 | 0.000 | 0.054 | 21 | 0.357 | 0.643 | 0.000 | 0.074 | 21 | 0.000 | 1.000 | 0.000 | 0.000 | 21 | 0.000 | 0.143 | 0.857 | 0.000 | 0.054 |
| MB1 | 302 | - | - | - | - | 32 | 0.969 | 0.031 | 0.000 | 0.022 | 32 | 0.203 | 0.797 | 0.000 | 0.050 | 32 | 0.000 | 1.000 | 0.000 | 0.000 | 32 | 0.000 | 0.031 | 0.969 | 0.000 | 0.022 |
| TBN | 343 | - | - | - | - | 22 | 0.977 | 0.023 | 0.000 | 0.023 | 22 | 0.114 | 0.886 | 0.000 | 0.048 | 22 | 0.000 | 1.000 | 0.000 | 0.000 | 22 | 0.000 | 0.000 | 1.000 | 0.000 | 0.000 |
| **Hackensack River** | | | | | | | | | | | | | | | | | | | | | | | | | |
| NK | 192 | - | - | - | - | 16 | 0.531 | 0.469 | 0.000 | 0.088 | 16 | 0.531 | 0.469 | 0.000 | 0.088 | 16 | 0.031 | 0.938 | 0.031 | 0.043 | 16 | 0.000 | 0.031 | 0.969 | 0.000 | 0.031 |
| PR | 202 | - | - | - | - | 19 | 0.526 | 0.474 | 0.000 | 0.081 | 20 | 0.350 | 0.650 | 0.000 | 0.075 | 20 | 0.000 | 0.975 | 0.025 | 0.025 | 16 | 0.000 | 0.031 | 0.969 | 0.000 | 0.073 |
| BC | 208 | - | - | - | - | 24 | 0.646 | 0.354 | 0.000 | 0.069 | 24 | 0.000 | 0.167 | 0.833 | 0.000 | 0.054 | 0.000 | 0.975 | 0.025 | 0.025 | 24 | 0.042 | 0.938 | 0.021 | 0.035 | 0.054 |
| VL | 210 | - | - | - | - | 78 | 0.538 | 0.462 | 0.000 | 0.040 | 78 | 0.353 | 0.641 | 0.006 | 0.038 | 33 | 0.015 | 0.894 | 0.091 | 0.038 | 34 | 0.000 | 0.206 | 0.794 | 0.000 | 0.049 |
| HG | 219 | 5 | 1.000 | 0.000 | 0.000 | 30 | 0.883 | 0.117 | 0.000 | 0.041 | 30 | 0.317 | 0.683 | 0.000 | 0.060 | 25 | 0.020 | 0.940 | 0.040 | 0.034 | 30 | 0.000 | 0.217 | 0.783 | 0.000 | 0.053 |
| CB | 223 | - | - | - | - | 34 | 0.882 | 0.118 | 0.000 | 0.039 | 34 | 0.309 | 0.691 | 0.000 | 0.056 | 9 | 0.000 | 0.944 | 0.056 | 0.054 | 20 | 0.000 | 0.250 | 0.700 | 0.050 | 0.068 |

**Table 1.** mtDNA haplotype frequency, Mdh-A, Ldh-B, Pgm-A, and Pgi-A gene frequencies for the sites sampled. (N) - sample size. Distance estimated along the shoreline from Tuckerton, NJ (TT)

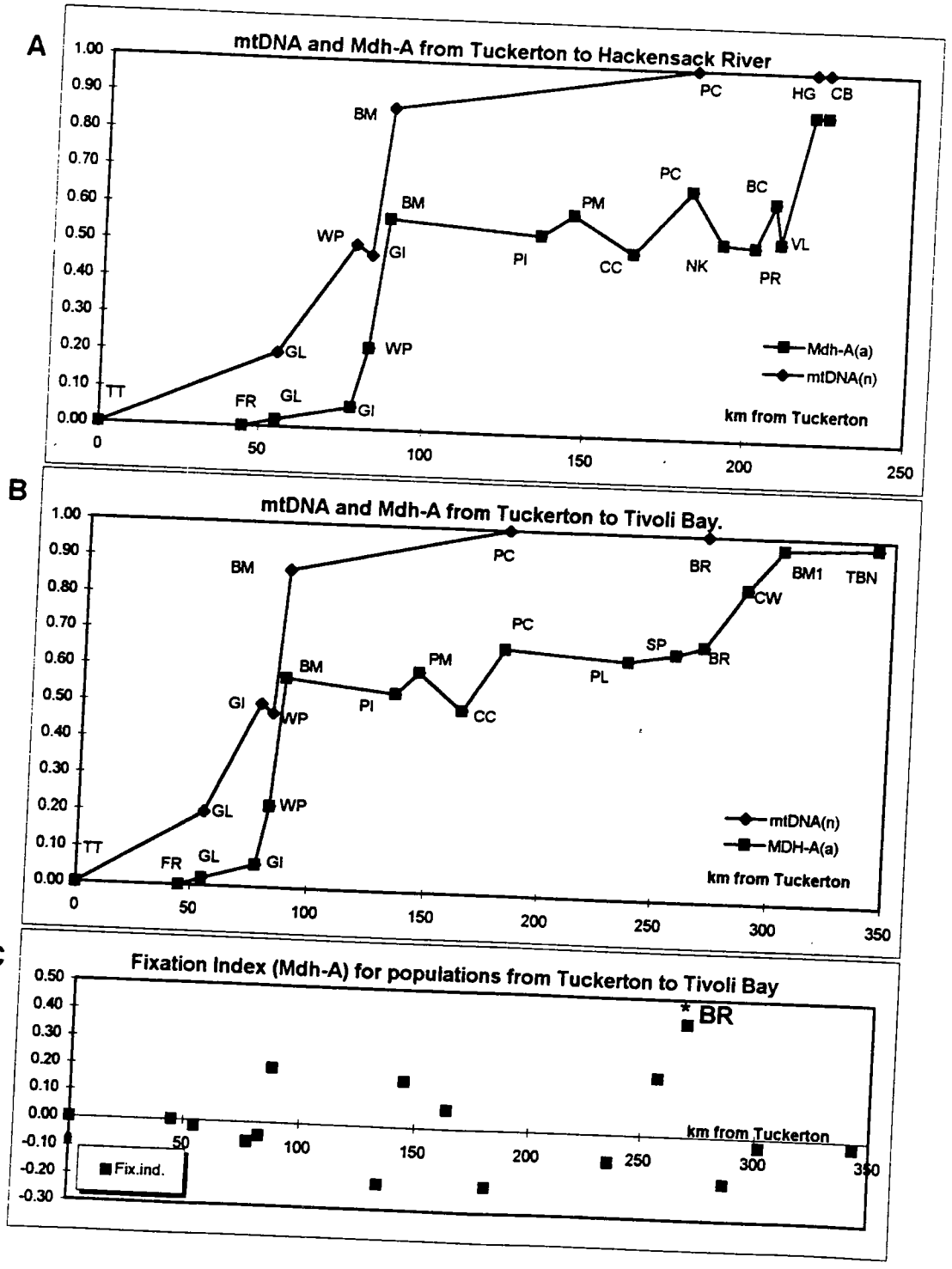



**Figure 3 A:** Mdh-A$^a$ and mtDNA$^n$ frequency from Tuckerton to Coles Brook on the Hackensack River; **B:** Mdh-A and mtDNA frequency from Tuckerton to Tivoli Bay on the Hudson River; **C:** Fixation Index for Mdh-A allele for populations from Tuckerton to Tivoli Bay. Star indicate significant ($p<0.05$) heterozygote deficiency at Iona Island estuary (BR), analyzed by $\chi^2$ test.



old fish. Each subsample was analyzed by $X^2$ -test separately, and significant heterozygote deficiency was detected for overwintered fish only ($X^2=4.222$, 1 df, $p=0.04$), and not for the young-of-the year mummichogs ($X^2=0.602$, 1 df, $p=0.438$) **(Fig. 4).**

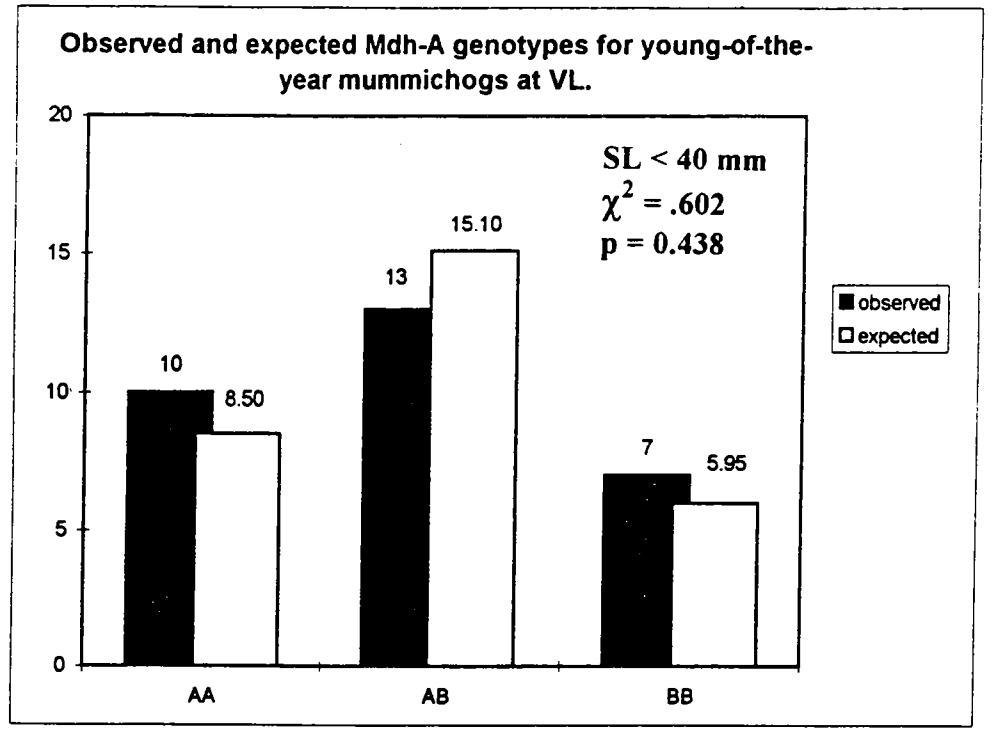
***F. h. macrolepidotus-F. diaphanus.***

At Marlboro-2 (**MB2**), and in South Tivoli Bay (**TBS**) both species were caught simultaneously, together with a number of young fish (30-50 mm SL) with appearance of *F. heteroclitus*, but which were revealed electrophoretically to be hybrids between *F. h. macrolepidotus* and *F. diaphanus*. No $F_1$ hybrids, but a number of backcrosses were found in both locations, probably because of small sample size (**Figs. 5** and **6**). Surprisingly, the hybridization zones between these two spp. are very narrow - at sites **MB1** (500 m downstream from **MB2**) neither *F. diaphanus* nor hybrids were found in 20 fish studied.

## Discussion

It is widely accepted that hybrid zones are natural laboratories which provide insight on processes of speciation and which can reveal the nature and effects of differences among incipient species (Barton and Hewitt, 1989). Surveys of mtDNA over the past few years revealed significant structuring of populations of many fish species (Chapman et al., 1993).

Population genetic analysis indicates three distinctive groups of *F. heteroclitus* populations which inhabit the area covered in our study. Being almost identical by appearance, they are different genotypically by mtDNA and Mdh-A frequency and produce distinctive branches in cladistic analysis **(Fig. 7)**.



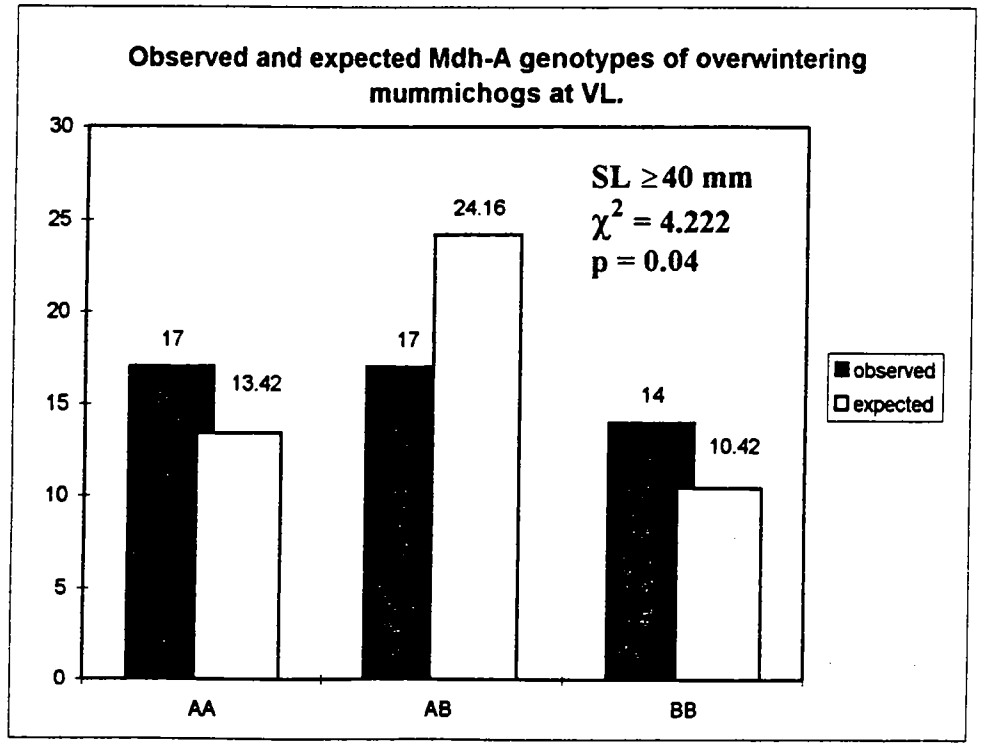
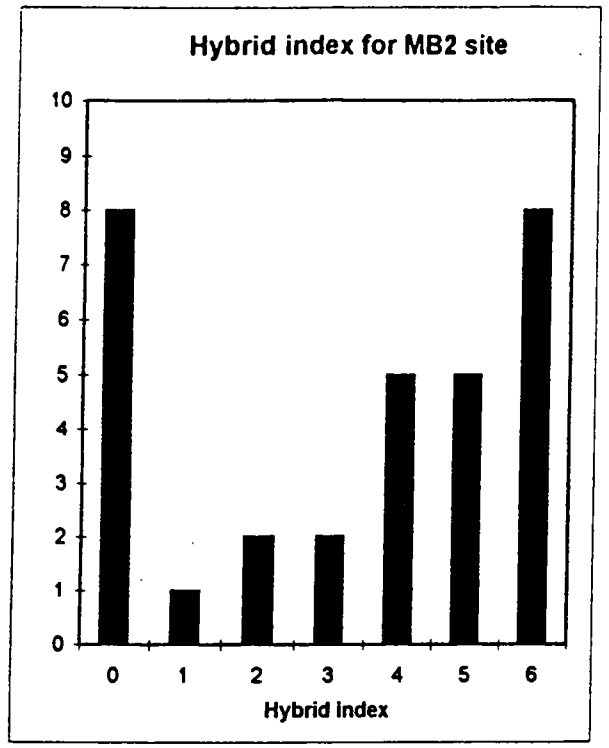



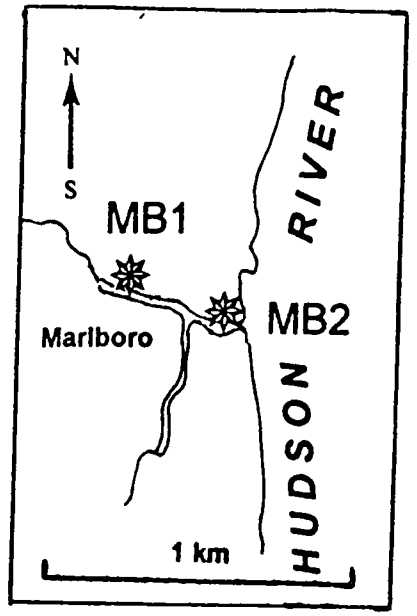

**Figure 4.** Mdh-A genotypes distribution for two age classes (young-of-the-year and 1-3 years old) in mummichogs from Vince Lombardi Rest Stop (VL) on Hackensack River. Deficiency of heterozygotes in overwintered fish may indicate differential mortality of *intermedius/macrolepidotus* hybrids during the first winter after hatching.



| Site | km | Chi-square | DF | P | Fixation index (F) |
|---|---|---|---|---|---|
| **New Jersey- Raritan Bays** | | | | | |
| TT | 0 | 0.289 | 1 | 0.591 | 0.059 |
| FR | 45 | - | - | - | - |
| GL | 55 | 0.099 | 3 | 0.992 | -0.020 |
| GI | 78 | 0.294 | 3 | 0.961 | -0.071 |
| WP | 83 | 0.021 | 1 | 0.884 | -0.049 |
| BM | 88 | 3.726 | 1 | 0.054 | 0.199 |
| PI | 135 | 0.878 | 1 | 0.349 | -0.208 |
| PM | 145 | 0.743 | 1 | 0.389 | 0.167 |
| CC | 164 | 0.154 | 1 | 0.695 | 0.067 |
| PC | 182 | 1.153 | 1 | 0.283 | -0.205 |
| **Connecticut** | | | | | |
| NW | 269 | 2.483 | 1 | 0.115 | 0.256 |
| NH | 318 | 0.035 | 1 | 0.851 | -0.034 |
| **Hudson River** | | | | | |
| PL | 236 | 0.109 | 1 | 0.742 | -0.099 |
| SP | 257 | 1.429 | 1 | 0.232 | 0.213 |
| BR | 269 | 5.177 | 1 | 0.023 | 0.409 |
| CW | 287 | 0.476 | 1 | 0.490 | -0.167 |
| MB1 | 302 | 0.016 | 1 | 0.898 | -0.032 |
| TBN | 343 | 0.000 | 1 | 1.000 | -0.023 |
| **Hackensack River** | | | | | |
| NK | 192 | 0.379 | 1 | 0.538 | 0.122 |
| PR | 202 | 2.948 | 1 | 0.086 | 0.367 |
| BC | 208 | 0.642 | 1 | 0.423 | -0.184 |
| VL | 210 | 4.222 | 1 | 0.040 | 0.226 |
| HG | 219 | 0.442 | 1 | 0.506 | -0.132 |
| CB | 223 | 0.522 | 1 | 0.470 | 0.117 |

**Table 2**. Fixation Index and $\chi^2$ –test for deviation from Hardy-Weinberg equilibrium for the all populations studied.



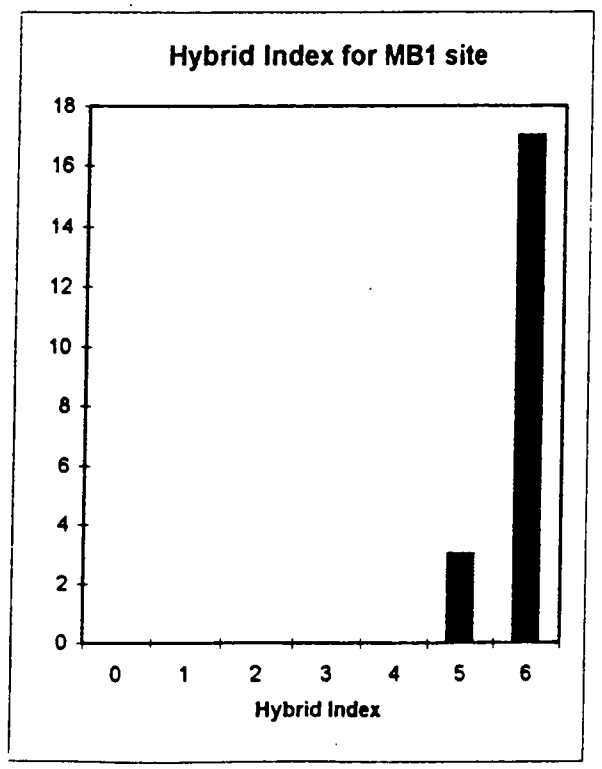
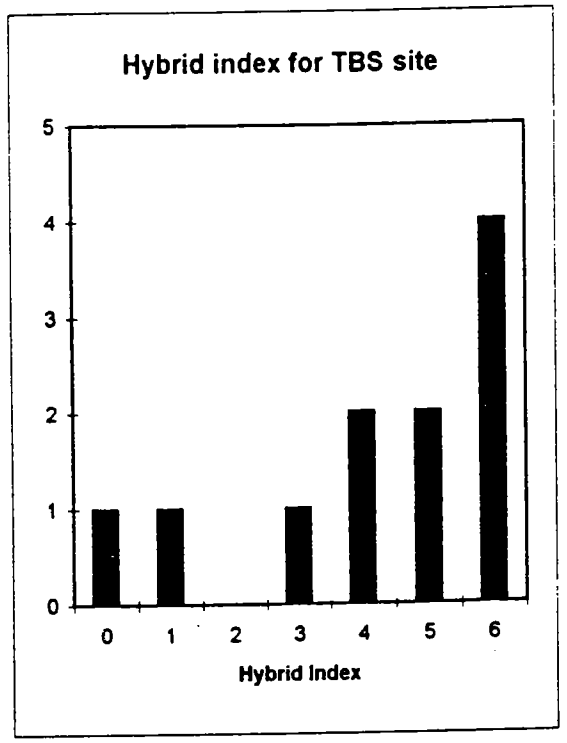
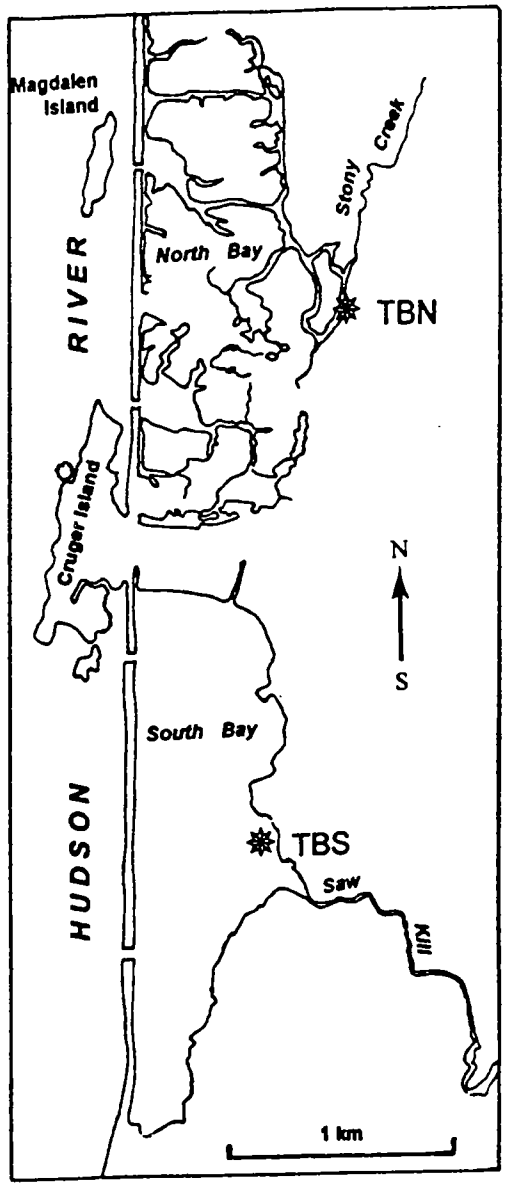




**Figure 5.** *F. heteroclitus - F.diaphanus* hybridization indexes in two populations on the Hudson River near Marlboro, NY. and a map with location of the sample sites.Index "0" indicates *F. diaphanus*, and "6" - *F.heteroclitus*. A few specimens with index "5" also belong to *F. heteroclitus* due to presence of weak polymorphism in Ldh-B allele.





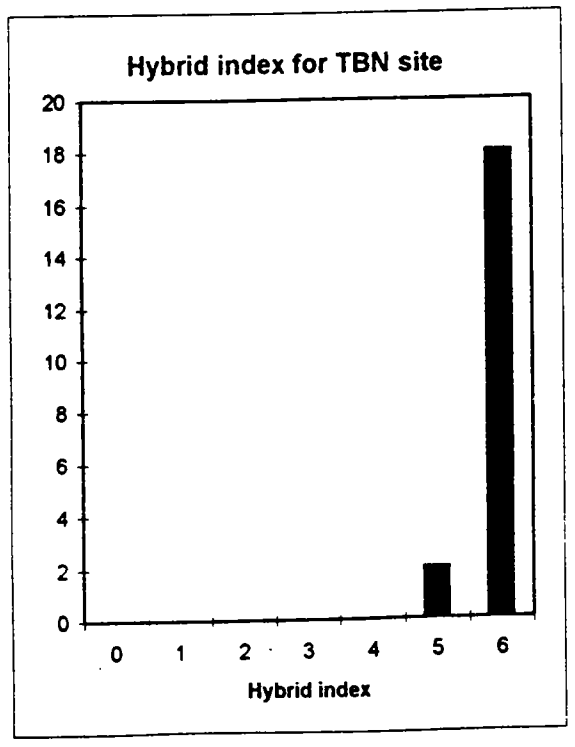




**Figure 6.** *F. heteroclitus - F.diaphanus* hybridization indexes for Tivoli Bays populations.

Populations from Tuckerton to Wreck Pond (NJ), including Barnegat Bay and the Manasquan River (sites **TT, FR, GL, GI, WP**) have a southern mtDNA haplotype (100% at **TT** to 60 % at **WP**) and Mdh-Ab allele (>94% from **TT** to **GI**, 80% at **WP**). According to Able and Felley (1986) and Smith et al., (1992), these populations represent *F. h. heteroclitus.*

In the upper estuaries (**HG** and **CB** on the Hackensack River and **CW, MB**, and **TB** on the Hudson River) and in Connecticut sites (**NW** and **NH**) mummichogs have very high frequency of Mdh-Aa (85-100%) and purely north mtDNA haplotype, and therefore represent the northern known counterpart - *F. heteroclitus macrolepidotus.*

For the area between these two genetically distinctive subspecies, cladistic analysis reveals the third group of populations. With a northern type of mtDNA (80% at BM and 100% in other sites), these populations have transitional frequencies of Mdh-A alleles, and therefore are highly polymorphic at this locus (50-75% for Mdh-Aa). To facilitate further discussion, we propose subspecies name *Fundulus heteroclitus intermedius* ssp.n. (as an intermediate between *heteroclitus* and *macrolepidotus*) for the fish caught in Shark River (**BM**), Raritan Bay (**PI, PM, PC**) and lower parts of estuaries to Vince Lombardi Rest Stop (Ridgefield, Borough, NJ) on the Hackensack River (**PR, BC, VL**), and to Iona Island (**PL, SP, BR**) on the Hudson River.

The presence of northern mtDNA patterns south from the *heteroclitus/intermedius* boundary indicates that the recent subspecies' distributions are not stable, and supports the idea that both taxa have been moving northward, concordant with climatic changes since the last glaciation (Powers et al., 1986). A very low frequency of Mdh-A$^a$ beyond this boundary raises the question of why selection against northern nuclear genes such as Mdh-A was much more severe than against mitochondrial genes. North and south Mdh-A alleles were studied for kinetic properties and thermal stability, and no significant differences were found, except that total MDH-A enzyme concentration in northern populations is higher. This effect may be explained by overall



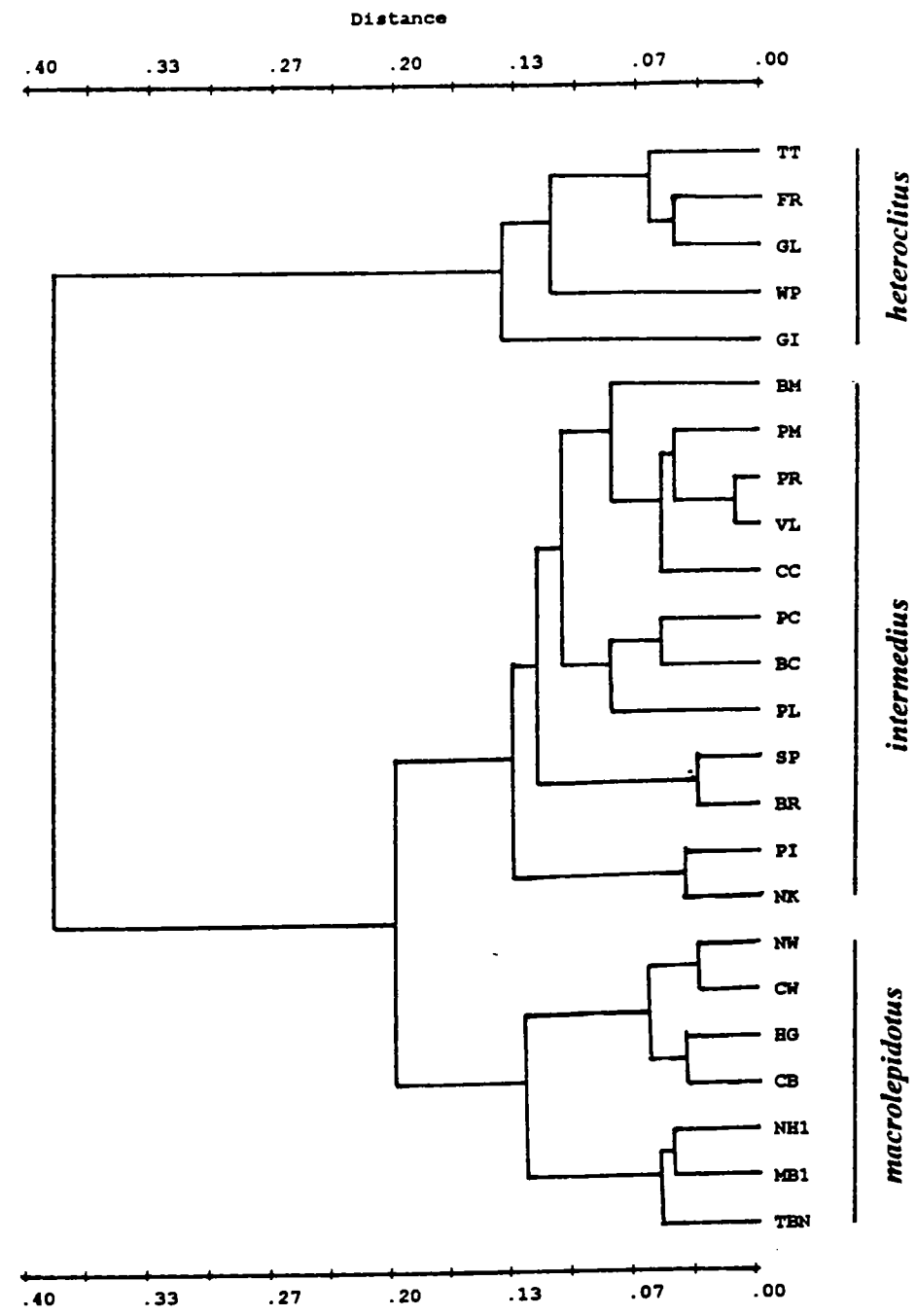



**Figure 7.**Cluster analysis of *F. heteroclitus* populations using unweighted pair group method. Coefficient used: modified Rogers distance (Wright,1978)