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An Analysis of the Genetic Differentiation in Disjunct Populations of *Campostoma oligolepis*, the Largescale Stoneroller

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UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

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PROJECT TITLE: An Analysis of the Genetic Differentiation in Disjunct Populations of Campostoma oligolepis, the Largescale Stoneroller

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

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Date: 10 May 1999

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Mentors:

Dr. David A. Etnier

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Purpose

This research project was created in order to look at the genetic variation that underlies the morphological differences noted between the Ozarks and the Tennessee/Mobile drainage systems in *Campostoma oligolepis*.

Abstract

The taxonomy of stoneroller fish (family Cyprinidae, genus *Campostoma*) is poorly understood, despite the fact that they are generally widespread and common in eastern North America. Prior morphological studies dealing with multiple characters suggest that there are five valid species of *Campostoma*, but this has not been adequately proven in any major publications.

Campostoma oligolepis, the Largescale Stoneroller, is found in eastern North America in three disjunct populations. There have been scale size differences noted between the Ozark highland population and the Mobile/Tennessee River drainages. In order to look at the genetic differences in this and other *Campostoma* species, specimens were collected in 1998 from these two populations of *Campostoma oligolepis*, as well as from populations of *Campostoma anomalum*, *Campostoma oligolepis* from the Conasauga River (Mobile drainage), and *Campostoma anomalum* x *oligolepis*. Scale counts were made and muscle samples were taken for genetic study. Allozyme analysis included looking at the differentiation between these two populations, and comparing this to differences is other species in this genus.

Seven variable loci were resolved. The patterns which emerged from the genetic data supports that hypothesized by Etnier from his morphological data. *Campostoma*

pullum is confirmed as a valid species. *Campostoma oligolepis* and *Campostoma anomalum* are more closely related taxa, with *Campostoma pullum* being a sister taxon to those species. Additionally, it is noted that *Campostoma oligolepis* shows some genetic variation between the two disjunct populations, but not to the degree observed between species.

This reveals that despite the usefulness of scale counts to distinguish between some species of stonerollers, it is not an accurate means to predict the phylogeny of the genus. Use of a suite of morphological characters or a genetic study is the best way to examine phylogeny.

Introduction

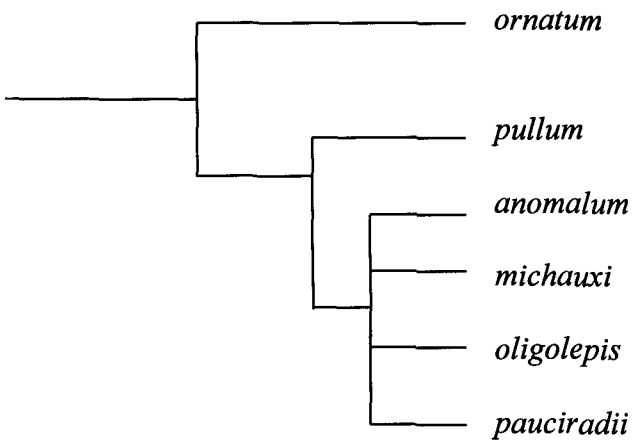
The genus *Campostoma* is a widespread and common group of fishes in the family Cyprinidae. They are distinguished from other genera in this family by an underslung mouth with a lower mandible modified into a cartilaginous shelf that is used to scrape algae off of rocks, and a long looped intestine which coils around the swim bladder which accommodates the difficulty of digesting algae (Page and Burr, 1991). The genus is actually named for this unusual mouth, with *campo* meaning curved and *stoma* meaning mouth (Etnier and Starnes, 1993). Despite their abundance throughout their ranges, the taxonomy of *Campostoma* is surprisingly poorly known. Some ichthyologists recognize five species, summarized in **Table 1**.

Table 1. Summary of morphological data for *Campostoma* – taken from Etnier's work

Campostoma species	Internasal Tubercles	Anal Fin Pigment	Size	Pharyngeal Teeth	Scales
anomalum	3-3	+	big	1,4-4,1	medium
oligolepis	0-0	0	big	1,4-4,1	large
ornatum	3-3	+	small	0,4-4,0	smallest
pauciradii	3-3	+	small	1,4-4,1	large
pullum	3-3	+	small	0,4-4,0	small
michauxi	3-3	??	big	1,4-4,1	small

michauxi is a recently published taxon, currently believed to be *Campostoma anomalum michauxi*.

David A. Etnier has hypothesized the following cladogram from the morphological data in **Table 1** to show the phylogenetic history of *Campostoma*:



Campostoma ornatum would therefore be the most primitive species in the genus. This is supported by the fact that it is the only species in this genus which does not feature an intestine which coils around the swim bladder (Page and Burr, 1911).

The ranges of the species examined in this thesis are illustrated below.

Figure 1. *Campostoma oligolepis*

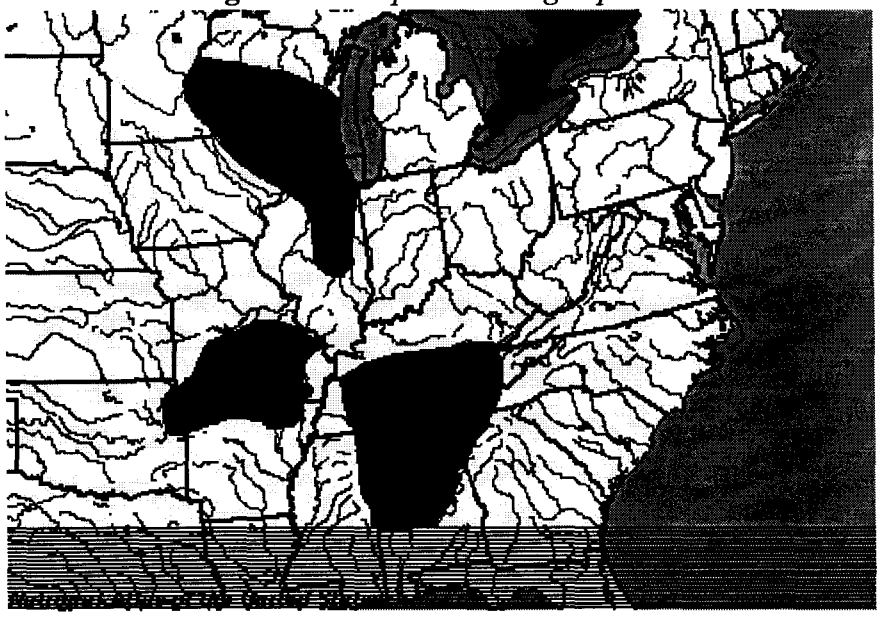


Figure 2. *Campostoma anomalum*

Figure 3. *Campostoma pullum*

Methods

Fishes were collected by bag seine in several localities (see **Table 2**)

Table 2. Collection sites.

Locality	Species Collected	Site	Date	Collectors	Used for allozymes?
Locality 1	*oligolepis, pullum*	Logan's Creek, Reynolds Co., MO	20 and 25 July 1998	AWJ, MOFEP	No
Locality 3	*oligolepis* x *anomalum*	Little Pigeon River, Sevier Co., TN	22 October 1999	DAE	Yes
Locality 4	*anomalum*	New River of Big South Fork, TN	21 October 1998	DAE	Yes
Locality 5	*oligolepis, pullum*	Current River, Van Buren, Carter Co.,MO	15 October 1998	ALM, AWJ	Yes
Locality 6	*oligolepis*	Conasauga River at 411 Bridge, Polk Co., TN	15 November 1998	DAE, AWJ	Yes

(Locality 2 is intentionally missing)
AWJ=Andrew W. Jones; ALM=Audrey L. Mayer; DAE=Dr. David A. Etnier; MOFEP=Caleb Putnam, Michelle Beck, Andrea Spender, Shane Aumiller, Mike Libsch and Duane White

Fishes taken at Locality 1 were placed on ice, then their scales were counted along the lateral line on the left side of the fish, as well as circumferentially, in front of the dorsal fin. These counts are later referred to as Lateral Line (LL) and Pre-dorsal Circumferential (PDC) counts. They are necessary for identification of *Campostoma oligolepis* and *Campostoma pullum* where they are sympatric (Localities 1 and 5). Additionally, the following were taken: eye, liver, and heart tissues, and muscle punches. The samples taken were then frozen in liquid Nitrogen. The fishes themselves were placed in formalin. Unfortunately, the muscle and tissue samples were all placed together in one aluminum foil package, so I was unable to separate the muscle sample for use in allozyme analysis. For the remainder of the localities (3 through 6), fish were brought to Dr. Etnier's lab alive, and then frozen in a standard refrigerator freezer. They were later transferred to a deep freezer in Dr. McCracken's lab (-80 to –120°C). When muscle samples were to be taken, the fish were unfrozen in groups of ten to fifteen. As each fish unthawed enough to be separated from the others, its scales were counted as described above.

Once the scales are counted, scales were cleared away from the right side of the fish in the shoulder region. A 5mm biopsy punch was used to take several muscle punches – half were placed in 1.5mL plastic tubes and stored in the deep freezer; half were placed in 95% ethanol and sent to Arizona State University to be used for mitochondrial DNA analysis by a graduate student there.

Proteins were then isolated from the frozen muscle samples by adding an equal volume of grinding buffer to each muscle sample, and then centrifuging at 14,000g for 2 to 3 minutes. The supernatant was extracted and placed in 0.5mL tubes.

Allozyme analysis was conducted with 10% horizontal starch gels (approximately 42 grams of potato starch and 425 milliliters of gel buffer). Small pieces of paper called "wicks" were placed in the muscle samples (which were thawed out enough to wet the wicks), and then placed in a slit towards one end of the gel. The gels, with a maximum of 25 individuals per run, were then incorporated into the gel setup and power was supplied as close to 50 milliAmperes as possible (to prevent the gels from overheating, therefore denaturing the proteins) for 2.5 to 6 hours, depending on the nature of the gel buffer.

At the conclusion of the running time, the gel was removed from the setup, the wicks were removed, and the nonessential regions of the gel were removed for ease of handling. The remaining gel was then sliced horizontally into four pieces. Each piece received a different protein stain, and was kept at 37°C. Once the proteins had reacted enough to be scorable, the protein stain was removed, and a small amount of Fixer solution added to maintain the products.

Gels were scored with the most cathodal allele labeled A, and the most cathodal locus labeled 1 (if necessary).

These procedures have been used by graduate students working in the McCracken lab, and are roughly equivalent to those described in Murphy et al. (1996).

Results and Conclusions

Multiple combinations of allozyme gel buffers and protein stains were tried. Included were thirteen stains and six buffers. Of the thirteen protein stains, seven were not resolvable on the systems I tried, one was monomorphic (showed no variation) and six showed polymorphisms. One (PGI) actually showed two polymorphic loci. The latter six were used for the genetic data in this project.

Scorable gels were recorded on paper, and often photographed as well. Each allele was summed up to provide gene frequencies for each population.

General Protein

This stain is used to visualize several tetramer products (Hillis, Moritz, and Mable, 1996). Four proteins were visible, but only the most cathodal product showed variation within my samples. The following gene frequencies were observed:

	A	B
oligolepis TN	100	0
oligolepis MO	93.75	6.25
pullum	86.67	13.33
anomalum	100	0
introgressed	100	0

Campostoma pullum is a more divergent form (as is suggested by the morphological data) and bears some affinities to *C. oligolepis* from Missouri. This supports the hypothesis that the Ozarks population of *C. oligolepis* is the older population, from which the other populations are derived. The fact that *C. oligolepis* from Tennessee and *C. anomalum* show the same genotype does not, however, support this notion, unless they have simply experienced convergent evolution. With gene frequency differences this small, it is hard to draw conclusions with any certainty.

Gene frequencies for this locus do not shed any light on the parentage of the introgressed individuals – they are identical to both *C. oligolepis* from Tennessee and *C. anomalum*.

Superoxide Dismutase (SOD) was often visible on this stain, but did not reveal any polymorphisms. In Buth and Burr (1978), this stain was monomorphic across all species except the more primitive *C. ornatum.*

Malate Dehydrogenase (MDH)

MDH stains for a dimer (Hillis, Moritz, and Mable, 1996). In my samples, it was revealed to be a pair of alleles that interacted. The following gene frequencies were recorded:

	A	B	C
oligolepis TN	0	55	45
oligolepis MO	0	51.67	48.33
pullum	0	77.5	22.5
anomalum	43.42	53.95	2.63
introgressed	43.75	56.25	0

This locus shows tremendous variation. Buth and Burr (1978) found MDH (both S-MDH and A-MDH) to be quite variable, with multiple alleles for S-MDH in both *C. oligolepis* and *C. anomalum*. In my samples, *C. pullum* show closer similarity to *C. oligolepis* (with it being a bit closer to *C. oligolepis* from Tennessee). *C. anomalum* has an additional locus which is not present in either of the other species, demonstrating a greater difference than expected from the morphological data.

The genetic divergence at this locus between the two populations of *C. oligolepis* is minimal, as compared to the differences between full species.

This locus shows great similarity between the pure *C. anomalum* and the introgressed individuals.

α Napthyl Acetate (αNA)

This stain is also known as a general Esterase Stain (Hillis, Moritz, and Mable, 1996). It was a monomeric protein. Because it revealed a different locus than did EST, I will maintain different names for them. The following gene frequencies were recorded:

	A	B
oligolepis TN	100	0
oligolepis MO	100	0
pullum	7.69	92.31
anomalum	96.43	3.57
introgressed	100	0

C. pullum is clearly the most divergent of the samples tested. It appears as though *C. anomalum* bears the most resemblance to *C. pullum*, as *C. oligolepis* has lost the B allele altogether.

The small number of introgressed samples provides a sampling error, as that the B allele could be present in low frequency, but could go unnoticed in this small sample.

In Rakocinski (1980), an Esterase protein stain was used that may have stained for the same locus as were seen by αNA in my samples. In Rakocinski's samples, it was noted to be fixed as BB in *C. oligolepis* and fixed as AA in *C. pullum* from Kilbuck Creek in northern Illinois (part of the Mississippi River drainage). Assuming that the alleles seen by Rakocinski were the same as I identified, then *C. pullum* from Missouri shows either some introgression with *C. oligolepis*, or maintenance of a more primitive locus. If the latter is true, then the lower frequency of the B allele in the anomalum

population may show itself as the next "branch" from the *C. oligolepis/anomalum/pauciradii* clade.

Esterase (EST-2)

Esterase stains for a monomer or dimer (Hillis, Moritz, and Mable, 1996) – in these samples, only the most anodal product showed any variation, which was a dimer. The following gene frequencies were seen:

	A	B
oligolepis TN	0	100
oligolepis MO	0	100
pullum	0	100
anomalum	0	100
introgressed	41.67	58.33

It is unusual that the introgressed individuals were the only ones to exhibit any variation. No variation was seen in *C. anomalum* from the New River, nor was any seen in *C. oligolepis* from Tennessee. It is possible that the allele not represented in this sample may indeed be present in *C. oligolepis* from the lower Tennessee River. Alternatively, the allele could exist in *C. anomalum* from the upper Tennessee River. In either case, neither population has come in direct contact with the populations I surveyed in a long time.

Phosphoglucomutase (PGM)

This stain shows a monomer (Hillis, Moritz, Mable, 1996). The following gene frequencies were encountered:

	A	B	C
oligolepis TN	1.66	98.33	0
oligolepis MO	0	96.43	3.57
pullum	0	31.82	68.18
anomalum		100 (n=3)	
introgressed			

I did not expect for this stain to work as well as it did, so not all specimens were scored for this locus.

C. pullum is the most divergent sample; the two populations of *C. oligolepis* are quite similar, with B being the most common allele. It is not necessarily very significant that there is a new locus (the A locus) present in the Tennessee *C. oligolepis* not found in any of the other populations. Appearance of a single allele within a population is not unexpected, particularly at the low frequency observed.

No conclusions may be reasonably drawn for *C. anomalum* due to the small sample size.

Buth and Burr's (1980) PGM results, when compared to mine, seem to indicate that their most cathodal allele was actually my B allele. This is because their *C. oligolepis* samples from Missouri were fixed at the most cathodal allele. My samples from this area were >90% B. With this in mind, it seems clear that their samples of *C. pullum* (identified as *C. anomalum* in their paper) were fixed for the C allele. My *C. pullum* samples were 68.18% C and 31.82% B, suggesting that the Missouri population is more closely related to *C. oligolepis* in MO than would be expected.

Phosphoglucoisomerase (PGI-1)

This protein is known to reveal a dimer product (Hillis, Moritz, and Mable, 1996). The following gene frequencies were observed:

	A	B
oligolepis TN	100	0
oligolepis MO	96.67	3.33
pullum	90	10
anomalum	100	0
introgressed	100	0

C. pullum is the most divergent population tested. As with PGM, a novel allele in a population of a species is not too unusual, and again it is present in low frequency.

The introgressed individuals have the same gene frequency as both parental species.

If Buth and Burr's (1978) results are compared with mine, the occasional B allele present in *C. oligolepis* from MO and in *C. pullum* are unexpected; their results showed both of the species to be fixed with respect to the A allele.

Phosphoglucoisomerase (PGI-2)

The same stain as used above revealed two sets of loci. This locus was actually represented as a dimer and a more anodal monomer with the same genotype. There was, however, one exception. Specimen 6.26 (*C. oligolepis* from Tennessee) was AB in the more cathodal monomer, and BB at the more anodal dimer. I have no explanation for this. For the purpose of calculating gene frequency, the AB combination was chosen.

The following gene frequencies were recorded:

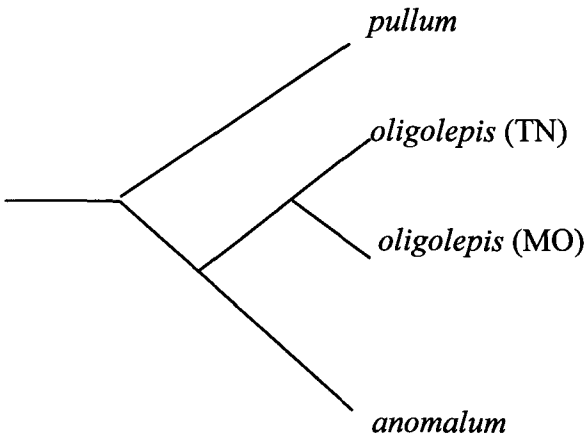
	A	B	C
oligolepis TN	34.38	65.52	0
oligolepis MO	0	100	0
pullum	31.82	59.09	9.009
anomalum	0	100	0
introgressed	0	75	25

This locus shows an unusual pattern among the species, in light of what other loci and morphological data show. At this locus alone, it appears as though *C. pullum* is most closely related to *C. oligolepis* from Tennessee (with a new locus appearing in *C. pullum*). *C. oligolepis* from Missouri and *C. anomalum* have the same genotype. One possible explanation is that the B allele may be the ancestral one, and that this locus may have undergone intense natural selection for *C. pullum* (resulting in the appearance of two new alleles) and *C. oligolepis* in Tennessee. This would support the idea that *C. oligolepis* originated in the Ozark highlands, and that it is most closely related to *C. anomalum*. Of course, it also may be hypothesized that the reverse would have occurred, wherein intense natural selection may have acted to "favor" the B allele.

The introgressed individuals show a superficial resemblance to *C. pullum*. Assuming that *C. oligolepis* and *C. anomalum* are the only parental species (as biogeography would clearly indicate), they are closer to *C. anomalum* because they lack the A allele.

Buth and Burr's (1978) work showed only occasional variation at this locus, and only in *C. oligolepis* and *C. pullum*. The A allele in my samples may be the same locus seen in *C. ornatum* in their paper, showing that *C. pullum* and *C. oligolepis* from Missouri are the older forms.

The net results from all of these gene frequencies is that *Campostoma pullum* is indeed a distinct taxon at the species level; it has consistently demonstrated more genetic divergence than any of the other populations. *Campostoma oligolepis* does not show any unexpected genetic difference between the populations in the Ozarks and in the Tennessee/Mobile drainages. Additionally, comparisons with data in Buth and Burr (1978) show that there is again only a moderate amount of genetic difference among the three disjunct populations (based on their Illinois samples). Evidence on the parentage of the introgressed individuals, though not consistent, seems to point towards a greater influence from the genes of *Campostoma anomalum* than from *Campostoma oligolepis*. Overall, this genetic evidence supports the phylogeny hypothesized by morphology, and suggests the following cladogram:



Mitochondrial DNA

The samples sent to Arizona State University are to be analyzed for their mitochondrial DNA sequences. Dr. Thomas Dowling has done some work with stonerollers, and currently has a graduate student (Evan Carson) who will be looking at these samples. At the time of completion of this thesis, the samples have not yet been examined.

Acknowledgements

I wish to thank Dr. David A. Etnier for his tremendous help in teaching me fish identification skills, and for helping me to mold this project towards completion. Dr. Gary F. McCracken was kind enough to let me use his laboratory for my research, and to help to find the best molecular technique for this research. Dr. Thomas Dowling provided startup advice on running allozymes for Stonerollers. Invaluable assistance in running and scoring allozyme gels was provided by Lisa Comeaux, Stan Guffey, Leslie Saidak, and Sunitha Vege.

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This thesis completes the requirements of the University of Tennessee Honors Program and the Threshold Program.

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Appendix

There are multiple versions of the recipes used for protein stains and gel buffers that have been published, and nomenclature for this type of research is not necessarily standardized among labs. Below are the recipes for all solutions and gels used in this research project. dH_2O stands for distilled water, and chemicals listed for a stain beneath the "====" symbol denoted unstable chemicals which should not be added until immediately before the stain is applied to the gel.

1. Gel Buffers

RSL Gel Buffer
Add 4 mL RSL Electrode Buffer(see below) to 400 mL total Volume with

Tris	14.53g
Citric Acid	4.20g

bring to 4L volume with dH_2O

CT "Morpholine" Gel Buffer
1:10 dilution of electrode buffer (see below)

Poulik Gel Buffer pH 8.7

Tris	36.84g
Citric Acid	4.2g

bring to 4L volume with dH_2O

Tris-HCl Gel Buffer pH 8.5

Tris	29.08g

adjust to pH 8.5 with dilute HCl
bring up to 4L volume with dH_2O

2. Tray (Electrode) Buffers

RSL Electrode Buffer

LiOH	10.08g
H3BO3	74.2g

bring to 4L volume with dH_2O

Borate Tray Buffer pH 8.2

Boric Acid	74g
NaOH	9.6g

bring to 4L volume with dH_20
[This is used for Poulik, Tris HCl and Tris EDTA Borate gels.]

CT "Morpholine" Electrode Buffer

Citric Acid	33.60g

bring to 4L volume with dH_2O
then adjust to pH desired with N-(3-aminopropyl)-morpholine

3. Protein Stains

AAT – Aspartate Aminotransferase

0.2M Tris-HCl pH 8.0	50mL
L-aspartic acid	50mg
alpha-ketoglutaric acid	100mg

readjust pH to 8.0 with NaOH
===

pyridoxal-5'-phosphate	0.5mg
Fast Blue BB Salt	100mg
Fast Garnet GBC Salt	200mg

stir with stir bar

ADH – Alcohol Dehydrogenase

0.5 Potassium Phosphate pH 7.0	5mL
dH_2O	40mL
95%EtOH	5mL

===

NAD	3mL
NBT	2mL
PMS	0.5mL

CK - Creatine Kinase

0.2M Tris-HCl pH 8.0	25mL
ADP	25mL
Glucose	900mg
Hexokinase	0.05mg
Phosphocreatine	50mg
0.1M $MgCl_2$	1.1mL

Agar overlay - 25mL H_2O/400mg agar
====

NADP	1.75mL
PMS	0.6mL
MTT	0.6mL

DIA

0.025M Tris-HCl pH 8.5	50mL
2,6-dichlorophenol-indophenol	5mg
NADH	10mg

incubate gel in the above for 30 minutes at 37°C
add MTT 0.75mL around the edges - not directly on the gel

EST - Esterases
[if gel is >ph7.0, then incubate at 37C in .5M Boric Acid]

Acetate pH 5.6	45mL
4-methyl-umbelliferate acetate	30mg

incubate in the dark at room temp for 5 minutes, then view on UV lamp

General Protein

napthol blue black	1g

stain-fixing solution	500mL

Leave on the gel for twenty minutes, then pour it off.
This solution may be reused.

IDH – Isocitrate Dehydrogenase

0.1M Magnesium Chloride	25mL
0.2M Tris-HCl pH 8.0	25mL
0.1M Isocitrate	1mL
====	
NADP	0.5mL
MTT	0.5mL
PMS	0.5mL

LDH – L-Lactate Dehydrogenase

0.2M Tris-HCl pH 8.0	40mL
0.1M Lithium Lactate, pH 8.0	8mL
=====	
NAD	1mL
NBT	1mL
PMS	1mL

MDH – Malate Dehydrogenase

0.2M Tris-HCl pH 8.0	50mL
MDH Stock Solution	8mL
====	
NAD	1mL
NBT	1mL
PMS	1mL

αNA - α Napthyl Acetate

Combine:	
α napthyl acetate	15mg
Acetone	0.75mL
dH_2O	0.75mL
Then add:	
Fast Blue RR Salt	50mg
0.05M Tris-HCl pH 7.0	50mL

PEP - Peptidases

Peptidases	20mg each
Peroxidase	20mg
O-dianisidine di-HCl	10mg
Snake Venom (*Bothrops ater*)	10mg
===	
0.2M Tris-HCl pH 8.0	50 mL
0.25M MnCl2	0.5mL

incubate at 37°C - view as IR

PGI – Phosphoglucoisomerase (also known as GPI – Glucose-6-Phosphate Isomerase)

0.2M Tris-HCl pH 8.0	45mL
Fructose 6-Phosphate	10mg
0.1M MgCl2	0.3mL
===	
G-6-PDH	0.3mL
NADP	0.6mL
PMS	0.1mL

MTT	1.2mL

PGM - Phosphoglucomutase

0.2M Tris-HCl pH 8.0	40mL
0.1M Magnesium Chloride	5mL
α-D-Glucose 1-Phosphate	100mg
====	
G6PDH	60u
NAD	1mL
NBT	0.5mL
PMS	0.5mL

4. Miscellaneous Solutions

Grinding Buffer

Tris	0.1211g
EDTA	0.037g

adjust pH to 6.8 with dilute HCl

NADP	0.4mL

bring to 100mL volume with dH_2O

Acetate pH 5.6

0.2M NaAcetate	27.2g
Acetic acid	12mL

bring to 1L volume with dH2O

MDH Stock Solution

Malic Acid	13.4g
dH_2O	40mL

dissolve with stirring, then slowly bring to pH 7.0 with:
2M Sodium Carbonate
bring volume to 100mL with dH_2O

Stain-Fixing Solution
1:5:5 ratio of glacial acetic acid; methanol; dH2O