Geographic Allozyme Variation In Kentucky Populations Of The *Desmognathus Fuscus* Complex (Plethodontidae)

A Thesis

Presented to

the Faculty of the College of Arts and Sciences

Morehead State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Russell M. Meadows

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Morehead State University, 1989

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Desmognathus fuscus has a long history of taxonomic confusion and instability with a long list of synonyms. Three forms of this species have been described from localities within Kentucky. Fifteen populations of the species complex D. fuscus were analyzed for 17 presumptive genetic loci by standard methods of horizontal starch gel electrophoresis. Genetic analysis provided an objective means of determining the number and geographic ranges of distinct forms of D. fuscus complex within Kentucky. Three distinct clusters resulted from an UPGMA analysis of electromorph frequency data, and indicated that each cluster represents a separate species. High levels of Nei's genetic distance values (D > 0.37) were calculated from between-species groups, and are concordant with genetic distance

values in other between-species comparisons of plethodontids (D > 0.15). Desmognathus. f. fuscus (Rafinesque) occurs in Kentucky in the Big Sandy, Licking, and Kentucky River drainages, and along the Ohio River westward to the Western Coal Field region. The second taxon was described as a subspecies by Rossman as D. f. conanti. It occurs in the Jackson Purchase region west of the Cumberland River, including the Mississippi Alluvial Plain. The third taxon, D. sp. nov., is a an undescribed species endemic to the Cumberland and Green River drainages of central Kentucky. Desmognathus sp. nov. shows low levels of heterozygosity in Kentucky populations, and low levels of genetic differentiation (D > 0.061) between populations that are separated by substantial geographic distance. This indicates that Kentucky D. sp. nov. may have gone through a population bottleneck during the last glaciation.

Accepted by: _____, Chairman

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INTRODUCTION

This study deals with population structure and genetic similarity in a species complex of the salamander genus Desmognathus that inhabits Kentucky. This complex is currently recognized as D. fuscus fuscus (Rafinesque) and D. f. conanti Rossman (Conant, 1975). The taxonomy of this complex is based on conservative morphological characters that are difficult to quantify and vary between and among populations and species. The result has been a long history of taxonomic confusion and instability with many synonyms for D. fuscus. Three forms have been described from localities within Kentucky. One was D. phoca Matthes (1855), which was synonymized with D. fuscus (Grobman, 1945). Barbour (1950) described a new subspecies, D. f. welteri from Big Black Mountain, Harlan Co., Kentucky, and indicated that this form occurs in many counties on the Cumberland Plateau and in Warren Co., which is part of the Mississippian Plateau. The Warren Co. site represents an outlying population removed from the known range by approximately 180 km. Barbour and Hays (1957) determined D. f. welteri to be the only form of D. fuscus inhabiting Kentucky and that it occurred essentially statewide. Rossman (1958) described D. f. conanti from Livingston Co., further complicating the already confused systematics of D. fuscus. Several authors (Juterbock, 1984; Caldwell and Trauth, 1979; Conant, 1975) now recognize D. welteri Barbour as a distinct species, separate from

D. f. fuscus. This species is believed to be restricted in range, within Kentucky, to the Cumberland Plateau. None of the currently described species can account for the large Desmognathus known to occur in Warren County. Barry Valentine (pers. comm.) has suggested that this is an undescribed species, bringing the count to three, the number of possible species of D. fuscus complex.

Allozyme electrophoresis is a technique used to study gene flow and the evolutionary history of closely related species. The number of such studies, now well over 1000 (Avise and Aquadro, 1982), clearly attests to the utility of electrophoretic methods in assessing the amount of genetic variation in natural populations. With this technique genetic affinities among populations and species can be measured with a variety of coefficients that quantify genetic similarities (Nei, 1978), genetic structure of populations (Hendrick, 1983; Wright, 1965), ages of divergence events between species (Maxson and Maxson, 1979; Sarich, 1977), and phylogenetic histories (Sites et al., 1984). Several electrophoretic studies have dealt with population structure and systematics of plethodontid salamanders, and proven useful in exposing cryptic species (Karlin and Guttman, 1986; Tilley and Schwerdtfeger, 1981; Tilley et al., 1978; Highton and Webster, 1976).

Evidence is provided to show the occurrence of three distinct genotypes within Kentucky. The undescribed form will herein be referred to D. sp. nov. It is not the intent of this study to

describe the new form, but to determine the number and geographic ranges of distinct genotypes of salamanders currently classified as D fuscus.

MATERIALS AND METHODS

Sample collection and preparation

Populations from 15 localities in Kentucky were sampled between January 1984 and December 1986 (Appendix 1, Fig. 1). Specimens were collected under rocks and logs along streams and springs in the hilly eastern region of the state; from wet areas near entrances to caves in the Mississippian limestone regions of central Kentucky, and under logs and leaf debris at the edge of wooded swamps in the Jackson Purchase region of western Kentucky. After capture, specimens were held in gallon size, zip-lock freezer bags filled with damp leaves and transported alive in ice-filled coolers to the laboratory at Morehead State University, Morehead, Kentucky, for electrophoretic analyses. Transport storage time varied from one to five days. In the laboratory, specimens were stored at 5° C in plastic trays lined with damp towels. Storage time varied between populations, but did not exceed two weeks, and had no effect on the amount or activity of allozymes extracted from the salamanders (Richard Highton, pers. comm.). Specimens were euthanized by drowning in degassed water prior to removal of viscera (excluding gonads) and ventral abdominal musculature. Carcasses were individually tagged, fixed in 10% formalin for 24 hours, rinsed in tap water for 24 hours, and preserved in 70% isopropyl alcohol. Tissue extracts were homogenized in equal volumes of grinding solution (Selander et al., 1971) using

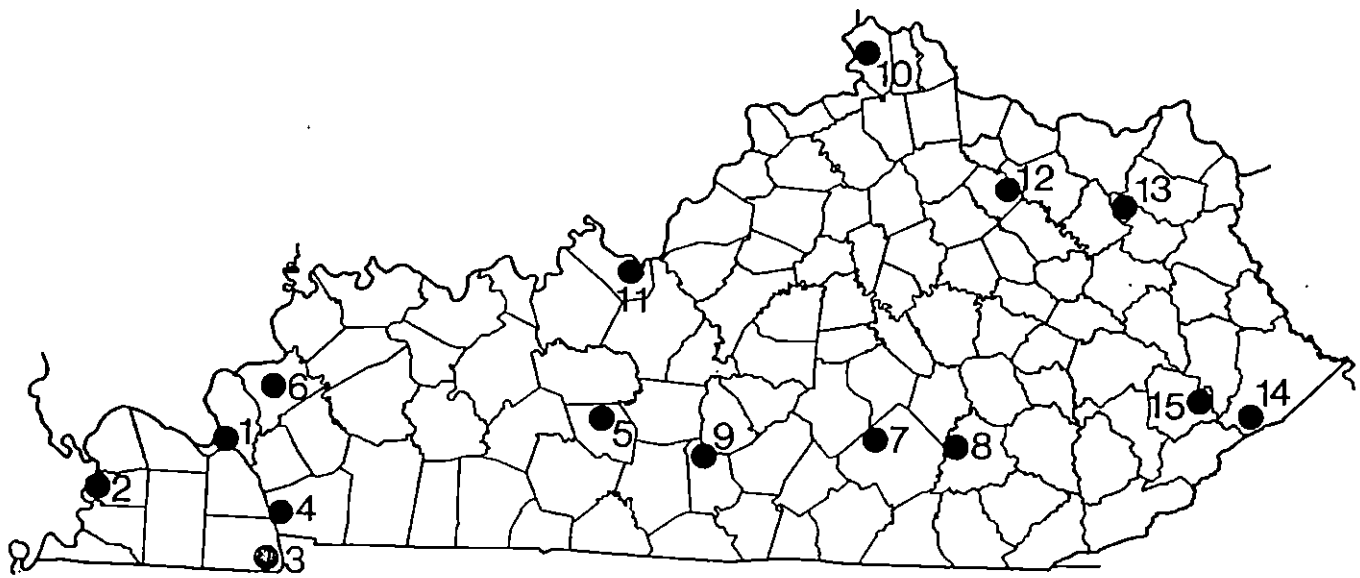

Figure 1. Localities of populations used in electrophoretic analyses.

Fisher 30 ml glass homogenizing tubes powered by a vertical drill press. Homogenates were centrifuged for 20 minutes at 2,000 g. Supernatants of soluble proteins were stored in Fisher 2 ml screwcap cryogenic vials and frozen in liquid nitrogen (-196° C) for later electrophoretic analysis.

Electrophoresis

Allozymes were resolved by horizontal starch gel electrophoresis with methods described by Selander et al. (1971), and Harris and Hopkinson (1976). The enzymes and buffer systems used to resolve them are shown in Table 1. First attempts to design a gel mold resulted in a mold 10 cm long x 20 cm wide x 2 cm deep which carried a large amount of current for a given voltage potential (i.e. high wattage). This resulted in heat outputs of approximately 30 watts which over-heated the gels and caused loss of allozyme activity, gel shrinkage, or in some cases, melting of the gels near the cathodal sponge. Since starch gels behave similarly to metallic resistors, the amount of current flow can be cut in half by doubling the length of the gel or halving its cross sectional area. Both of these were accomplished (using the gel design in Fig. 2), thus decreasing the wattage by 75%. This mold is 22 cm long x 20 cm wide x 1 cm deep (inside measurements) and constructed from 1/4 inch plexiglass.

Gels were made by suspending 50 g of Sigma starch in 400 ml of gel buffer (12.5%) using a magnetic spin bar. The starch solution

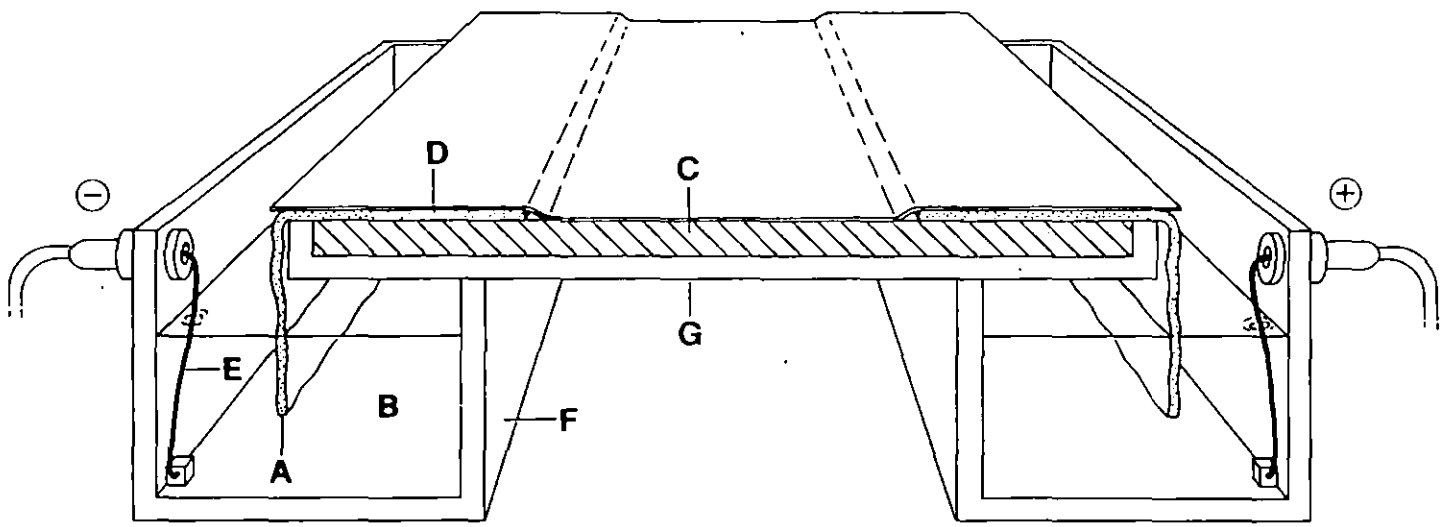

Figure 2. Electrophoretic apparatus. Key: A, sponge wicks; B, bridge buffer; C, starch gel; D, plastic sheet; E, platinum electrode; F, buffer tank; G, gel mold.

was brought to a boil in a one litre vacuum flask, degassed under vacuum to remove bubbles, then poured into a gel mold. Gels were immediately covered with plastic wrap to prevent a thick skin from forming, then allowed to cool to room temperature before being refrigerated until use. If the starch solution was not fully suspended before heating, the resulting gels were lumpy.

Samples were inserted into the gels, along a line cut across the gel 6 cm from the cathodal end, on 10 x 5 mm pieces of Whatman No. 3 MM filter paper saturated with soluble proteins. These were blotted dry before being loaded into the gel to prevent band smearing. Each gel of the four buffer systems contained a replicate set of samples (Table 1). After electrophoresis, gels were sliced into 2 mm thick slices and stained according to the isozymes being resolved. Appendix 2 gives the stain recipes used to identify the isozymes in Table 1.

Each gel contained samples from populations having variant electromorphs so each locus from a population could be compared to known electromorphs for that locus. For each locus, the most anodally migrating electromorph was designated A, the second-most B, and so forth. Loci were numbered in the same manner. Electrophoresis resolves only those amino acid substitutions that confer charge alterations on polypeptides. Electromorph variants too similar in motilities to be consistently separated were considered as a single variant, thus conferring a conservative estimate of genetic

Table 1. Buffers used in electrophoresis and proteins assayed on each buffer.

Buffer	Proteins assayed
Lithium hydroxide Gel pH 8.2 Tray pH 8.1	Lactate dehydrogenase (Ldh-1) Malic enzyme (Me) Hexose-6-phosphate dehydrogenase (H-6-Pdh)
Poulik Gel pH 8.7 Tray pH 8.2	Alpha-naphol acetate esterase (Est-1, Est-2) Fumarate hydratase (Fum) General protein (Pt-1) Lactate dehydrogenase (Ldh-2)
Tris-citrate pH 6.7 Gel pH 6.7 Tray pH 6.3	Malate dehydrogenase (Mdh-1, Mdh-2) Aspartate aminotransferase (Aat-1, Aat-2) Superoxide dismutase (Sod)
Tris-citrate pH 8.0 Gel pH 8.0 Tray pH 8.0	Glutamate dehydrogenase (Gdh) Isocitrate dehydrogenase (Icd-1, Icd-2) Aspartate aminotransferase (Aat-1) Glucose-6-phosphate dehydrogenase (G-6-Pdh)

diversity between population comparisons.

Data analyses

Electrophoretic data were based on 17 presumptive genetic loci (Table 1). Individual genotypes were recorded from electromorph patterns and allele frequencies calculated from genotype frequencies. Nei's (1978) unbiased estimates of normalized identity of alleles (I), genetic distance (D), and average population heterozygosity (H), including average alleles per locus (A), were estimated from allele frequencies. Phylogenetic relationships were analyzed according to the unweighted pair-group arithmetic average method UPGMA (Sneath and Sokal, 1973).

RESULTS

Patterns of electromorph variation

Geographic patterns in electromorph frequency distribution generally agree with patterns observed in other electrophoretic studies of D. fuscus (Karlin and Guttman, 1986; Tilley and Schwerdtfeger, 1981). The first pattern is random variation in electromorph frequency with no detectable clines or geographic regularities. The second pattern appears to be interpopulational differentiation in electromorph frequencies along a cline. The third, and most significant, pattern is fixed electromorph differences between any groups of populations.

Tilley and Schwerdtfeger (1981) observed six of 18 allozymes surveyed (33%) to be fixed or virtually fixed for the same electromorphs in all populations sampled. Karlin and Guttman (1986) also observed 33% (seven of 21) of the allozymes surveyed to be fixed for the same electromorphs in all populations.

Of the 17 presumptive genetic loci surveyed in this study, six (Icd-2, Gdh, G-6-Pdh, Mdh-2, Pt-1, and Sod) or 35% were fixed for the same electromorphs in all populations. The electromorph frequencies for 11 polymorphic allozymes are reported in Table 2.

Electromorph frequency patterns for four proteins (Me, Aat-1: Fig. 3, Mdh-1, and H-6-Pdh) demonstrated random variation, as shown in Tilley and Schwerdtfeger's (1981) first pattern. For example,

Table 2. Variation in electromorph frequencies of polymorphic loci within and among samples of *Desmognathus*.

		D. f. conanti				*D. sp. nov.*					*D. f. fuscus*					
Population:		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Locus	N:	14	13	18	18	11	10	7	10	18	24	18	5	14	16	10
Ldh-1	a	1.0	1.0	1.0	1.0											
	b										1.0	.93	.50	.93	.94	.83
	c					1.0	1.0	1.0	1.0	1.0		.07	.50	.07	.06	.17
Ldh-2	a	1.0	1.0	1.0	1.0				.70		.67	.25				
	b					1.0	1.0	1.0	.30	1.0	.23	.22		.64	.45	.58
	c										.10	.53	1.0	.36	.55	.42
Est-1	a	.04	.42													
	b	.96	.58	1.0	1.0											
	c							.07	.75		.15	.12	.30	.21	.14	
	d					1.0	1.0	.93	.25	1.0	.85	.88	.70	.79	.86	1.0
Est-2	a	1.0	1.0	1.0	1.0											
	b					1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Fum	a	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		.71			.17	
	b										1.0	.29	1.0	1.0	.83	1.0
Me	a										.75	.50				
	b	.96	.69	1.0	1.0	1.0	1.0	1.0	.95	1.0	.25	.50	1.0	1.0	1.0	1.0
	c	.04	.31						.05							

Table 2. (Continued).

		D. f. conanti				*D. sp. nov.*					*D. f. fuscus*					
Population:		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Locus	N:	14	13	18	18	11	10	7	10	18	24	18	5	14	16	10
H-6-Pdh	a							.14	.05							
	b	1.0	1.0	1.0	1.0	1.0	1.0	.86	.95	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Iod-1	a										1.0	.89	1.0	.64	.56	.95
	b	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		.19		.36	.44	.05
Aat-1	a		.27		.31	1.0	1.0	1.0	1.0	1.0				.04		
	b	1.0	.73	1.0	.69						1.0	1.0	1.0	.96	1.0	1.0
Aat-2	a					1.0	1.0	1.0	1.0	1.0						
	b	1.0	1.0	1.0	1.0						1.0	1.0	1.0	1.0	1.0	1.0
Mdh-1	a		.08	.75												
	b	1.0	.92	.25	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

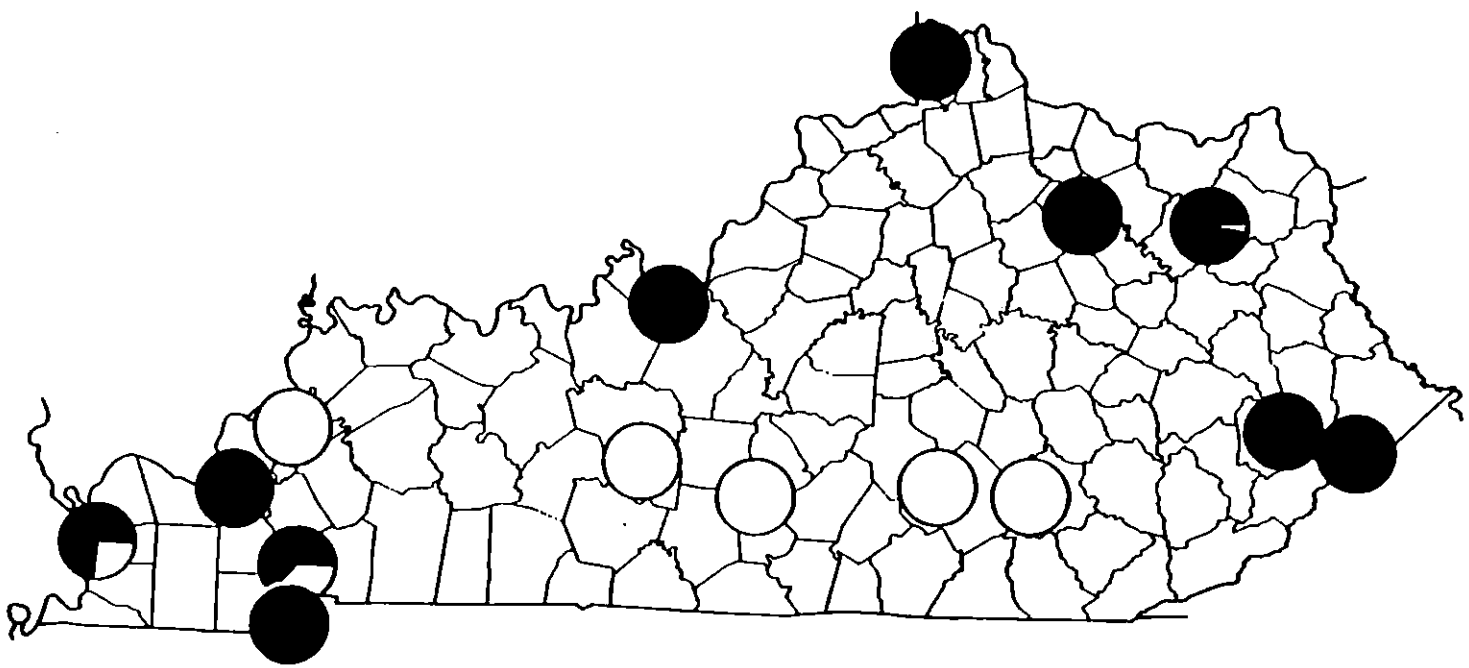

Figure 3. Frequencies of the Aat-1 alleles; open circles are allele a, closed circles are allele b.

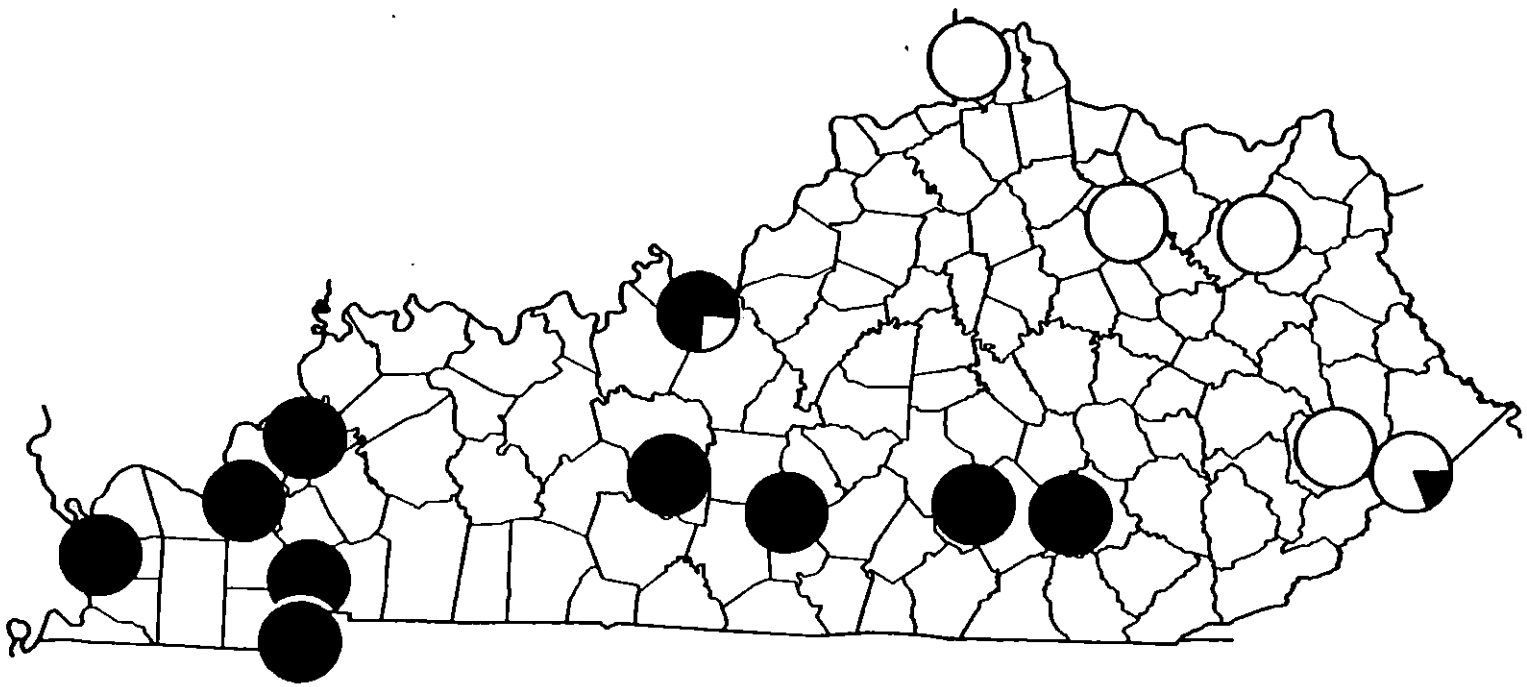

Figure 4. Frequencies of the Icd-1 alleles; open circles are allele a, closed circles are allele b.

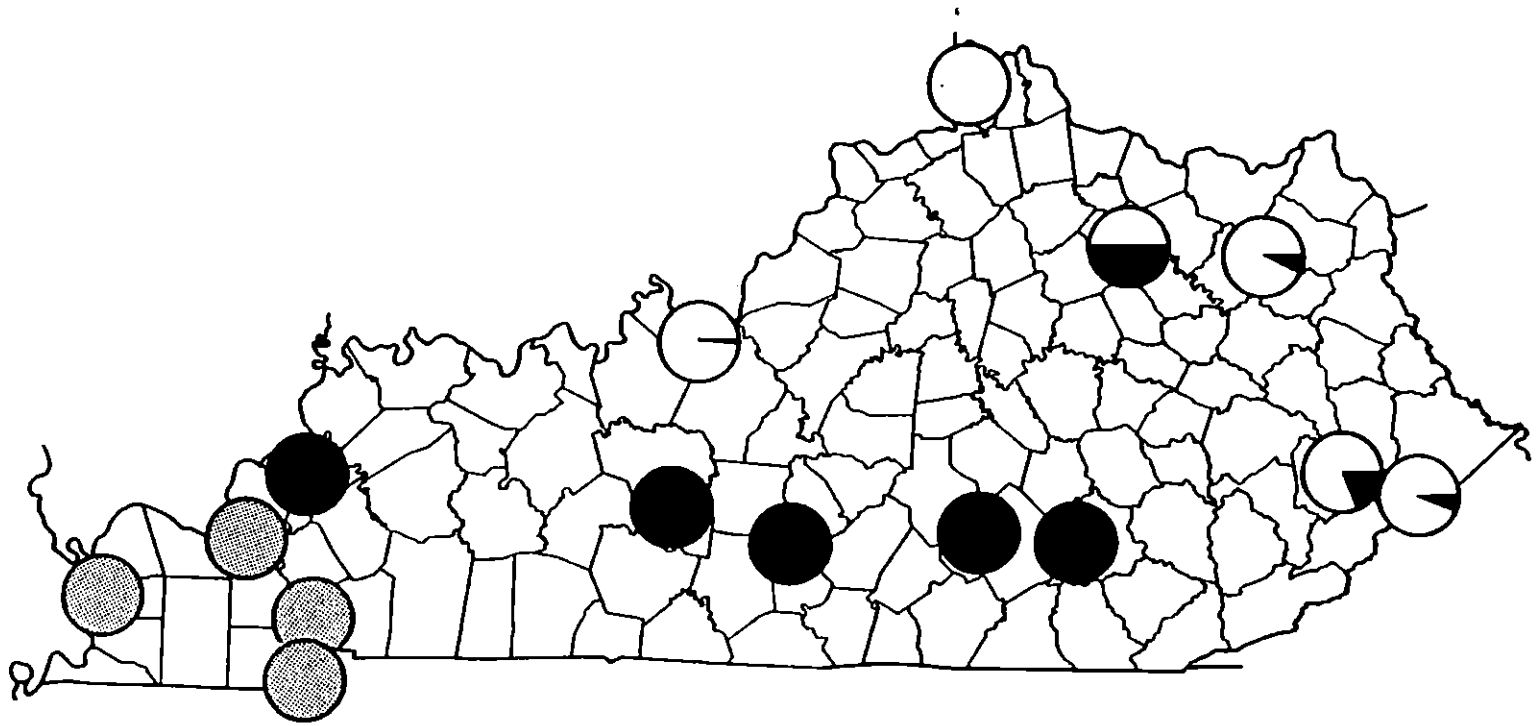

Figure 5. Frequencies of the LDH-1 alleles; stippled circles are allele a, closed circles are allele b, open circles are allele c.

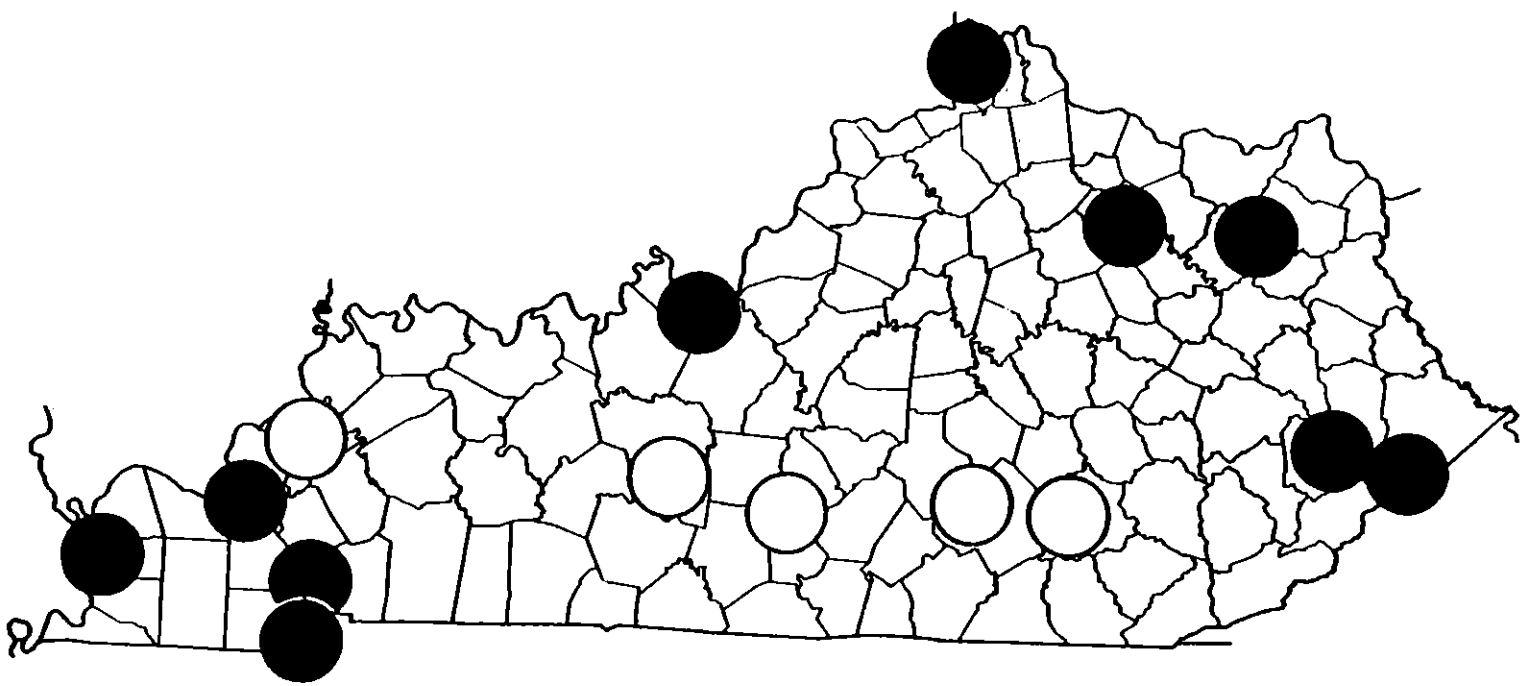

Figure 6. Frequencies of the Aat-2 alleles; open circles are allele a, closed circles are allele b.

three electromorphs were resolved for Me, but the same electromorph prevailed through most populations (Me^b, Table 2). Some populations (e.g., populations 7 and 8, for H-6-Pdh^a; populations 10 and 11, for Me^a) were polymorphic for rare electromorphs. Karlin and Guttman (1986) similarly assigned Me, Aat-1, and Mdh-1 to this type of pattern.

The remaining seven proteins (Ldh-1, Ldh-2, Est-1, Est-2, Aat-2, Icd-1,and Fum; Table 2) showed a complex pattern of electromorph variation between groups of populations representing the three taxa. Icd-1 (Fig. 4) showed a pattern of abrupt differentiation in electromorph frequencies between populations 5-9 (D. f. fuscus) and 10-15 (D. sp. nov.), this follows Tilley and Schwerdtfeger's (1981) second pattern.

The third and most significant pattern is fixation for opposite electromorphs in groups of populations representing different species. Comparisons of populations representing D. f. conanti and D. sp. nov. showed five proteins (Ldh-1: Fig. 5, Ldh-2, Est-1, Est-2, and Aat-2: Fig. 6; Table 2) that were fixed or virtually fixed for opposite electromorphs. There were some exceptions to fixation; population 8 had a high frequency of the Ldh-2^a allele of D. f. conanti (Table 2). This could have resulted from random genetic drift due to a past hybridization or from the ancestral stock. Four proteins (Ldh-1, Est-1, Est-2, and Aat-1; Table 2) were fixed for opposite electromorphs between populations of D. f. conanti and

D. f. fuscus. When D. sp. nov. was compared with D. f. fuscus two proteins (Aat-1: Fig. 3 and Aat-2: Fig. 6; Table 2) were fixed for alternative electromorphs. Fum also showed near fixation of alternate electromorphs, except that population 11 of D. f. fuscus had a high frequency of the Fum^a electromorph of D. sp. nov. (Table 2).

Average population heterozygosity (H) values ranged from 0.00 to 0.13, and were consistent with other H values reported for Desmognathus (Tilley and Schwerdtfeger, 1981). Heterozygosity values were consistently low for populations 5-9 (H ranged from 0.00 to 0.061); three of the populations (populations 5, 6, and 9; Table 2) were monomorphic for all loci.

Population cluster analysis

Nei's (1978) unbiased genetic distance (D), calculated from electromorph frequencies, ranged from 0.000 (population 5 vs. 6) to 0.516 (population 3 vs. 13) (Table 3). Comparisons of D values among populations 1-4 were consistently low ($D < 0.05$). Similarly, small D values were obtained for comparisons among populations 5-9 ($D < 0.07$) and populations 10-15 ($D < 0.12$). However, when interspecies groups were compared there was a marked increase in D values. For example, the average genetic distance between D. sp. nov. and D. f. fuscus was 0.371, range 0.30-0.50. Larger D values were obtained for other comparisons among the three taxa (Table 3).

Three clusters shown in the phenogram in Fig. 7 resulted from a UPGMA analysis (Sneath and Sokal, 1973) of the D values in Table 3. The cophenetic correlation was 0.96, and showed a good fit of the D values to the phenogram. The clusters represent the three taxa of Desmognathus. The first cluster represents populations 1-4 of D. f. conanti, the second cluster represents populations 5-9 of D. sp. nov., and the third cluster represents D. f. fuscus.

Table 3. Nei's genetic identities and distances for 15 populations of *Desmognathus fuscus* complex. Normalized identity of genes (I) above the diagonal and genetic distance (D) below the diagonal for all pairs of samples. Genetic heterozygosity (H) and average number of alleles per locus (A) are given at bottom of table. Low D values (D < 0.15) indicate within species population comparisons while high D values (D > 0.15) indicate between species population comparisons.

	D. f. conanti				*D. sp. nov.*					*D. f. fuscus*					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1		.984	.967	.995	.648	.648	.647	.663	.648	.668	.735	.668	.639	.721	.669
2	.016		.953	.985	.671	.671	.670	.688	.671	.669	.735	.657	.645	.711	.659
3	.034	.048		.961	.610	.610	.609	.623	.610	.627	.694	.629	.597	.681	.631
4	.005	.015	.040		.674	.674	.674	.689	.674	.655	.723	.657	.627	.710	.659
5	.434	.399	.494	.394		1.000	.999	.938	1.000	.629	.726	.680	.672	.743	.714
6	.434	.399	.494	.394	.000		.999	.938	1.000	.629	.726	.680	.672	.743	.714
7	.435	.400	.496	.395	.001	.001		.944	.999	.625	.722	.677	.669	.740	.710
8	.412	.374	.473	.372	.064	.064	.058		.938	.607	.721	.720	.650	.730	.677
9	.434	.399	.494	.394	.000	.000	.001	.064		.629	.726	.680	.672	.743	.714

Table 3. (Continued).

	D. f. conanti				D. sp. nov.					D. f. fuscus					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10	.404	.402	.468	.424	.463	.463	.470	.500	.463		.952	.910	.968	.930	.943
11	.307	.308	.365	.324	.321	.321	.326	.327	.321	.049		.927	.943	.960	.946
12	.403	.420	.463	.420	.386	.386	.390	.328	.386	.094	.076		.895	.964	.972
13	.448	.439	.516	.467	.398	.398	.403	.431	.398	.033	.059	.111		.934	.931
14	.328	.341	.384	.343	.298	.298	.302	.315	.298	.073	.041	.036	.068		.990
15	.401	.418	.461	.418	.337	.337	.343	.390	.337	.058	.056	.028	.072	.010	
H	.009	.090	.023	.026	.000	.000	.024	.061	.000	.067	.126	.060	.089	.099	.055
A	1.118	1.235	1.059	1.059	1.000	1.000	1.118	1.235	1.000	1.235	1.412	1.118	1.294	1.294	1.176

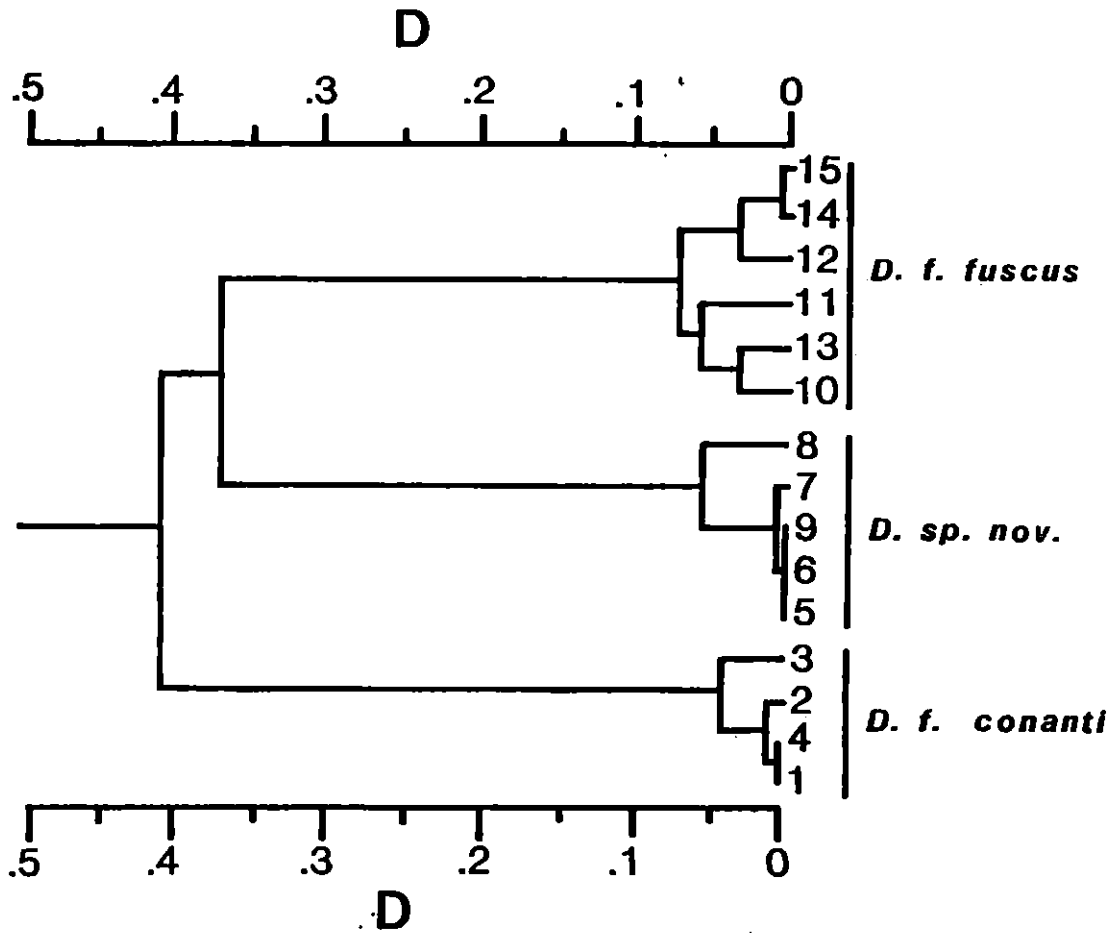

Figure 7. UPGMA phenogram of D values of samples from three species of the Desmognathus fuscus complex. Cophenetic correlation coefficient is 0.96.

DISCUSSION

Three taxa of D. fuscus complex occur in Kentucky. Desmognathus f. fuscus (Rafinesque) occurs in the Big Sandy, Licking, and Kentucky River drainages, and along the Ohio River westward to the Western Coal Field region. The second taxon, Desmognath sp. nov., is endemic to the Cumberland and Green River drainages of central Kentucky. The third taxon was described from Livingston Co., Kentucky, by Rossman (1958) as D. f. conanti. This form inhabits the Jackson Purchase region west of the Cumberland River, including the Mississippi Alluvial Plain. Population 1 is from the type locality of D. f. conanti (Appendix 1).

Populations from the three taxa differ dramatically in electromorph frequencies, and are fixed for alternative electromorphs in at least three proteins between any two taxa. This indicates that no gene exchange is occurring among the three taxa. Average genetic distances (Fig. 7) between the taxa are consistent with D values between other plethodontid salamanders (Karlin and Guttman, 1986; Highton and MacGregor, 1983). Maxson and Maxson (1979) estimated that plethodontid salamanders accumulate a Nei's genetic distance of about 1.0 every 14 million years. The average D value between D. f. fuscus and D. sp. nov. is 0.371, between D. f. fuscus and D. f. conanti is 0.403, and between D. f. conanti and D. sp. nov. is 0.426. The molecular clock estimate for the time of divergence of

D. f. conanti and D. f. fuscus is between 5.3 and 5.9 million years ago, or about mid-Pliocene. The phenogram (Fig.2) indicates D. sp. nov. and D. f. fuscus diverged shortly after the separation from D. f. conanti, approximately 4.9 million years ago.

Desmognathus sp. nov. shows low levels of heterozygosity in Kentucky populations, and low levels of genetic differentiation (D>0.061) between populations that are separated by substantial geographic distance (Table 3). This indicates that Kentucky D. sp. nov. may have gone through a population bottleneck during the last glaciation.

The description and life history of D. sp. nov., as well as, the taxonomic status of D. f. conanti will be the topics of later research.

Recent analyses of populations have determined D. f. fuscus and D. sp. nov. to be sympatric along a narrow contact zone in the Cumberland and Green River drainages in Jackson Co., Kentucky (unpubl. data).

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APPENDIX I

Population	Locality
1.	Livingston Co., 2.4 mi. south of the junction of US 60 and KY 453 on US 60.
2.	Carlisle Co., Laketon, 0.5 mi. north along old railroad track.
3.	Calloway Co., 1.9 mi. south of KY 121 on Winchester Rd., behind old quarry.
4.	Trigg Co., Stream 0.8 mi. south of KY 807 along Dover Rd.
5.	Edmonson Co., 3.9 mi. east of Nolin River Dam on KY 728 at Bylew Cave.
6.	Crittenden Co., spring run at end of Freedom Church Rd.
7.	Paulaski Co., Dumpling Cave south of Cave Hill Rd., ca. 1.6 mi. east of KY 70.
8.	Laurel Co., Falls City Rd. at Hawks Creek.
9.	Metcalfe Co., 2.1 mi. north of KY 70 on KY 1048, behind Seven Springs Church.
10.	Boone Co., Woolper Rd., 1.3 mi. north of KY 18.
11.	Meade Co., Morgan's Cave, ca. 0.5 mi. north of Rock Haven on Otter Creek Park Rd.
12.	Nicholas Co., Clay Farm Wildlife Management Area; 1.0 mi. from lower entrance on road leading to Licking River.
13.	Carter Co., Goodin Branch Rd., 1.6 mi. north of KY 504.
14.	Pike Co., Lower Pigeon Creek, off of KY 197 5.85 mi. east of KY 119/US 23.
15.	Knott Co., ravine on the north side of KY 7, 0.4 mi. south of the Jct. with KY 1091.

APPENDIX II

Allozyme stains

The following is a list of histological stains used to isolate the given proteins and references from which the stains were modified.

Aspartate aminotransferase (E.C. 2.6.1.1)

Distilled H_2O	200.0 ml
Alpha-Ketoglutaric acid	0.146 g
L-Aspartic acid	0.533 g
PVP-40	1.0 g
Na_2EDTA	0.20 g
Na_2HPO_4	5.68 g
To stain add:	
2.0 M Tris/HCl, pH. 8.0	25.0 ml
Substrate soln. (above)	25.0 ml
Fast garnet GBC*	100.0 mg

Incubate in the dark at 37° C.

Note: Incubate 20 min. at room temperature in stain soln. before adding the Fast Garnet salts.

Modified from Selander et al. (1971).

Esterase (E.C. 3.1.1.1)

0.2 M $NaH_2PO_4 \cdot H_2O$, pH.4.4	25.0 ml
0.2 M $Na_2HPO_4 \cdot 7H_2O$, pH 8.7	5.0 ml
Distilled H_2O	20.0 ml
Fast garnet GBC	20.0 mg
To stain add:	
Alpha-Naphthyl acetate	1.5 mg
Beta-Naphthyl acetate	1.5 mg

Incubate at room temperature.

Note: Dissolve the acetates in 1.0 ml of acetone before adding to the soln.

Modified from Harris and Hopkinson (1976).

Fumarate hydratase (E.C. 4.2.1.2)

0.2 M Tris/HCl pH. 8.0	50.0 ml
Fumaric acid	0.15 mg
Add just before use:	
Malate dehydrogenase (250 units/ml)	1.0 ml
1% Nicotinamide adenine dinucleotide (NAD)	1.0 ml
Nitro blue tetrazolium (NBT)	1.0 ml
1% MTT	1.0 ml
1% Phenazine methosulfate (PMS)*	0.3 ml

Incubate in the dark at 37° C.

*Note: Incubate one hour before adding PMS.

Modified from Brewer (1970).

Glucose dehydrogenase (E.C. 1.1.1.47)

0.1 M KH_2PO_4, pH. 7.0	50.0 ml
D-Glucose	9.0 g
1% NAD	2.0 ml
1% PMS	0.5 ml
1% NBT	0.5 ml

Incubate in the dark at 37° C.

Modified from Harris and Hopkinson (1976).

Glutamate dehydrogenase (E.C. 1.4.1.2)

Distilled H_2O	43.0 ml
0.5 M Potassium phosphate buffer, pH. 7.0*	16.0 ml
L-Glutamic acid	0.25 g
Add just before use:	
1% NAD	3.7 ml
1% PMS	0.25 ml
1% MTT	1.2 ml
1% NBT	1.2 ml

Incubate in the dark at 37° C.

*Note: Made from 0.5 M K_2HPO_4, pH. 8.8 and 0.5 M KH_2PO_4, pH. 4.1 with the pH. adjusted to 7.0 using 0.5 M KH_2PO_4.

Modified from Shaw and Prasad (1970).

Isocitrate dehydrogenase (E.C. 1.1.1.42)

0.2 M Tris/HCl, pH. 8.0	50.0 ml
0.2 M $MgCl_2 \cdot 6H_2O$	5.0 ml
0.1 M DL-Isocitric acid	3.0 ml
Add just before use:	
1% Nicotinamide adenine dinucleotide phosphate (NADP)	1.0 ml
1% PMS	0.5 ml
1% MTT	0.3 ml

Incubate in the dark at 37° C.

Modified from Selander et al. (1971).

Lactate dehydrogenase (E.C. 1.1.1.27)

Distilled H_2O	30.0 ml
0.2 M Tris/HCl, pH. 8.0	30.0 ml
0.5 M DL-Lactic acid	1.5 ml
Add just before use:	
1% NAD	2.0 ml
1% PMS	0.8 ml
1% NBT	0.8 ml

Incubate in the dark at 37° C.

Modified from Selander et al. (1971).

Malate dehydrogenase (E.C. 1.1.1.37)

0.2 M Tris/HCl, pH. 8.0	30.0 ml
2.0 M DL-Malic acid, pH. 7.0	8.0 ml
Add just before use:	
1% MTT	1.5 ml
1% PMS	0.8 ml
1% NAD	3.5 ml

Incubate in the dark at 37° C.

Modified from Selander et al. (1971).

Malic enzyme (E.C. 1.1.1.40)

0.2 M Tris/HCl, pH. 8.0	30.0 ml
2.0 M DL-Malic acid, pH. 7.0	10.0 ml
Add just before use:	
1% NBT	1.5 ml
1% NADP	1.5 ml
1% PMS	0.1 ml

Incubate in the dark at 37° C.

Modified from Selander et al. (1971).

Proteins (generic)

Comassie blue	0.20 mg
Acetic acid (glacial)	5.0 ml
Methanol	25.0 ml
Distilled H_2O	25.0 ml

Incubate at room temperature until the gel is dark blue; clear the gel in a fresh solution containing no Comassie blue until the background lightens.

Superoxide dismutase (E.C. 1.15.1.1)

0.2 M Tris/HCl, pH.8.0	50.0 ml.
1% MTT	5.0 ml.
1% PMS	5.0 ml.

Expose to light for several minutes then incubate at 37° C.

Modified from Harris and Hopkinson (1976).