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CHARACTERIZATION OF THE TREEFROG  
NULL ALLELE, 1991

by  
Sheldon I. Guttman

Department of Zoology  
Miami University  
Oxford, Ohio 45056

April 1992

PREPARED FOR THE  
FERNALD ENVIRONMENTAL MANAGEMENT PROJECT  
Westinghouse Environmental Management Company of Ohio  
P.O. BOX 398704  
CINCINNATI, OHIO 45239-8704

Under Contract DE-AC05-86OR21600  
**U.S. Department of Energy**  
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Approved:   
S.I. Guttman, Professor of Zoology

Approved:   
L.S. Farmer, Mgr., Environmental Mgt.

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## CHARACTERIZATION OF THE TREEFROG NULL ALLELE

## EXECUTIVE SUMMARY

Spring peeper (*Hyla crucifer*) tadpoles collected from the waste storage area during the Biological and Ecological Site Characterization of the Feed Materials Production Center in 1986 and 1987 appeared to be unique. A null (inactive) allele was found at the glucose phosphate isomerase (GPI) enzyme locus in significant frequencies (approximately 20%) each year; this allele did not appear to occur in the offsite sample collected approximately 15 km from the FEMP. Null alleles at this locus have not been reported in other amphibian populations; when they have been found in other organisms they have invariably been lethal in the homozygous condition.

Analyses in 1990 demonstrated that the null allele is found in populations up to 20 km from the FEMP; re-examination of gel photographs from Facemire et al. (1990) determined that the null GPI allele had been erroneously classified as absent from their Reily population sample. However, there is a marked decrease in frequency of the allele with increasing distance east and south from the FEMP. Decrease in frequency of the null allele is more gradual north of the site with nearly significant reductions not occurring until a distance of 17 km.

During 1991 sampling west of the FEMP as well as at distances greater than 20 km, along the remaining compass points, was conducted. This sampling regimen was established to confirm whether the frequency of this unique null allele continues to decline with increasing distance from the FEMP and vicinity, and to aid in determining the origination site of the null allele. Laboratory crosses were made between individuals heterozygous for the null allele to determine whether this null allele is lethal, when homozygous, in spring peepers.

The 1991 study indicated that the null allele is present in higher frequency over a much wider distribution than was previously anticipated. The allele is present at a frequency of 18% in a population of treefrogs at Fort Jefferson, 77 km north of the FEMP. In addition, sampling 70 km west of the FEMP documented the null allele at a frequency of 20% near Greensburg, IN. However, the allele continues to decrease both east (Wheeling, 2%) and south (Rice Run, 1%) of the site. Distributional data suggest that it is highly unlikely that the FEMP was the site of origin for the null allele.

None of the treefrogs collected over the past three years were homozygous for the null allele. Crosses between null heterozygotes failed to yield the predicted frequency of null homozygotes; in fact, no null homozygous offspring were found. These data appear to confirm the lethality of the homozygous null condition.

## CHARACTERIZATION OF THE TREEFROG NULL ALLELE

## INTRODUCTION

As part of our intensive year-long baseline ecological study (Facemire et al. 1990), we characterized the degree of genetic polymorphism and heterozygosity in selected Fernald Environmental Monitoring Project (FEMP) plant and animal populations using electrophoretic techniques. These data are being used as an indicator of stress by comparing populations on and off the FEMP property.

Anurans (frogs and toads) are ideal organisms for use as monitors of stress in freshwater environments. They breed and lay their eggs in water and the tadpoles spend from one to three months (depending on the species) developing in aquatic habitats; during the tadpole stage they are herbivores feeding predominantly on algae. Upon metamorphosis anurans become terrestrial carnivores. They may remain in the vicinity of their natal pond or disperse up to 10 km (Ewert 1969). However, during their first breeding season they demonstrate a remarkable fidelity to their natal site and most will migrate directly back to the pond in which they developed (Christein et al. 1979). Adults will continue to return to the same site year after year (Oldham 1967) and cases exist of individuals returning to the site of a natal pond long after it has been obliterated (Heusser 1960).

We studied treefrogs (spring peeper; Hyla crucifer) because they are one of the most abundant anuran species in the vicinity

of the FEMP and because they breed on-property. Spring peeper females each deposit approximately 900 eggs, egg hatch occurs in 6 days and tadpoles require about 45 days in water before metamorphosis (Gosner and Rossman 1960). First reproduction occurs at one year of age (Collins 1975) and the longevity record for a treefrog of this genus is 14 years of age (Bowler 1977).

The study of treefrogs on and in the vicinity of the FEMP began in 1986 when spring peeper tadpoles (N=80) were collected at the FEMP from an ephemeral pond, surrounded by mowed grass, within the waste pit area about 150 m south of Waste Pit 6 and compared with 20 tadpoles from an off-property population in Indian Creek County Park, Butler County, Ohio (Facemire et al. 1990). A significant, unique allele at the glucose phosphate isomerase (GPI) locus was found in the FEMP treefrog tadpoles. GPI is an essential glycolytic enzyme. The allele, GPI<sup>n</sup>, is a null (inactive under assay conditions). It was present in FEMP samples with a frequency of approximately 20% and was expressed only in heterozygous condition. This allele was not present in tadpoles from off-property yielding significant heterogeneity ( $p < 0.001$ ) between the FEMP and off-property populations at this locus. Guttman's (1985) recent review of the literature on biochemical population genetics of frogs and toads did not indicate any instance of a null allele at a glycolytic enzyme locus. Null alleles have occasionally been found at glycolytic enzyme loci in other organisms and they are often associated with disease (Spiess 1977). However, GPI homozygous nulls have

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been demonstrated to be lethal. Burkhart et al. (1984) analyzed null alleles in Drosophila and noted that GPI was the only essential locus among those studied; the null was lethal as a homozygote. Hydrops fetalis or immediate neonatal death of humans has recently been shown to be caused by GPI deficiency (Ravindranth et al. 1987).

In 1990, 783 additional Hyla crucifer were sampled at distances of up to 20 km from the FEMP (Guttman 1990). No individual was homozygous for GPI<sup>n</sup>. Data indicated that highest frequencies of GPI<sup>n</sup> consistently occurred in the immediate vicinity of the FEMP although populations occurring within an area approximately 15 km north of the property possessed the GPI<sup>n</sup> allele in frequencies ranging between 15%-22%. Resampling of the off-property population in Indian Creek County Park studied by Facemire et al. (1990) demonstrated that the null allele was present at that site but had been mis-identified on photographs of electrophoretic gels in the earlier investigation. Frequencies for GPI<sup>n</sup> decreased both north (12%) of this zone as well as southeast and south of the FEMP (5% and 7%, respectively).

The current study was initiated to determine whether this GPI null allele is lethal, when homozygous, in spring peepers. Also, a sampling protocol was implemented to determine whether a linear relationship exists between frequency of the null allele and distance from the FEMP and to attempt to determine the origination site of the null allele.

## METHODS

Five hundred and three adult Hyla crucifer were collected from 10 sites (Sites 6, 11, 17-22, 24, 27; Table 1, Figure 1). The first three sites had been sampled by Guttman (1990) but were recollected to obtain males and gravid females of appropriate genotypes for crosses (see below); data from these samples were included in population genotype determinations. Treefrogs were brought back to Miami University, toe-clipped with a unique number and maintained at 5C. Toes removed during marking were frozen at -70C to prevent loss of GPI activity. Males ceased calling after 29 April 1991; therefore, additional adults could not be collected. Three new sites (23, 25 and 26; Table 1, Figure 1) were sampled using only tadpoles. A total of 361 Hyla crucifer tadpoles were collected for this study.

Toes from individual treefrogs and whole individual tadpoles were homogenized manually with 0.25M sucrose 2% 2-phenoxyethanol in cold spot plates. The homogenates were then stored at -70C until analyzed by electrophoresis; this occurred within four days of homogenization.

Glucose phosphate isomerase was analyzed using a citric acid, 4-(3-aminopropyl)morpholine, pH 6.1 buffer (Clayton and Tretiak 1972) and 15% Sigma starch gel (Sigma Chemical Co., St. Louis), with histochemical procedures following Selander et al. (1971). After the gels were stained, the genotypic composition of each treefrog was determined and recorded. Genotypic data were analyzed using the BIOSYS-I computer program (Swofford and Selander 1981). Allele frequencies, deviation

from expected proportions assuming Hardy-Weinberg equilibrium conditions, amount of genetic variation, similarity matrices and chi-square contingency tests for interpopulation heterogeneity were performed. Results are discussed and interpreted relative to treefrog populations studied in 1986 and 1987 (Facemire et al. 1990) and 1990 (Guttman 1990). Prior to commencing the study, protocols were submitted and approved by WMCO.

Glucose phosphate isomerase genotypes were determined from toe clips of males and gravid females collected early in the breeding season. Appropriate adults were crossed; for each cross the following procedure occurred. The testis from a double-pithed male was removed, placed into a petri dish with 5 ml Holtfreter's solution (Rugh 1962), and then macerated. After 10 min, eggs were stripped into the solution from the appropriate gravid female, and the mixture gently agitated for 30 sec to ensure exposure of sperm to unfertilized eggs. Thirty min later, the petri dishes were filled with Holtfreter's solution and the dishes were placed in 1 L crystallizing bowls. Airstones provided aeration for the developing embryos and tadpoles. When tadpoles hatched and yolk supplies were exhausted, boiled lettuce was provided ad libitum. Approximately three weeks after hatching, tadpoles were of sufficient size for electrophoretic analysis and were frozen at -70C until used.

Selected adults were frozen at -70C after GPI genotypes were determined and maintained as vouchers; the remaining adults

were returned to the sites where they were collected and released.

## RESULTS

Five GPI alleles were detected in the 864 Hyla crucifer collected in the field; one (GPI<sup>e</sup>), present in low frequency in a single population (#20), represents an allele not observed by Guttman (1990). However, Guttman (1990) identified GPI<sup>d</sup> which was not found in the present study. Allele frequencies in the populations studied are listed in Table 2; the distribution of the null allele GPI<sup>n</sup> is mapped in Fig. 2. No individual sampled was homozygous for GPI<sup>n</sup>. These determinations reinforce genotypic analyses in Guttman (1990) and crossing data below; all evidence indicate that individuals possessing the GPI<sup>nn</sup> genotype do not survive more than a few weeks post-hatch.

The highest frequency of GPI<sup>n</sup> determined during this study occurred in the population at Lakengren (#19, Table 1), 43 km north of the FEMP. All sites within 80 km of the FEMP, except for the Kentucky population (#21) maintained GPI<sup>n</sup> in frequencies  $\geq 10\%$ ; in population #21 and eastern populations more than 125 km from the FEMP allele frequencies for GPI<sup>n</sup> decreased dramatically to 3% or less (Fig. 2).

Two types of contingency chi-square analyses were performed. The first considered all five alleles detected in the populations sampled during 1991; the second analysis pooled all alleles except GPI<sup>n</sup> into an "other" category and compared

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the frequencies of the null and "other" categories across populations.

Using all alleles, those samples collected within 20 km of the FEMP are significantly heterogeneous ( $p=0.01$ ; Table 3); the majority of the contribution to the high chi-square value derives from the low frequency of GPI<sup>a</sup> in the Booth Road (#17) sample and high frequency of GPI<sup>b</sup> at the Hilltop (#6) site. Significant ( $p<0.0001$ ) heterogeneity was apparent when those populations (#18-22) collected 30-45 km from the FEMP were compared; heterogeneity was due to the disparate distribution of all five alleles. In particular, the southern (#21) sample had GPI<sup>n</sup> present in low (0.01) frequency. Separation of the Hedgerow Road (#18) and Lakengren (#19) populations into a northern group maintained within-group heterogeneity ( $p=0.001$ ; Table 3) attributable to the presence of GPI<sup>b</sup> only at #19 and GPI<sup>a</sup> being three times more frequent in population #18 than the former site. Those populations (#23-27) sampled at distances greater than 70 km from the FEMP are also significantly heterogeneous ( $p<0.00001$ ) due to the high frequency of GPI<sup>n</sup> and GPI<sup>b</sup> in the western Greensburg (#23) and northern Ft. Jefferson (#24) samples compared to the eastern populations (#25-27); eastern populations tended to have high frequencies of GPI<sup>a</sup>. Comparison of only the eastern populations yielded data indicating these three populations are homogeneous at GPI.

Results differ slightly when all alleles except GPI<sup>n</sup> were pooled into an "other" category and the frequencies of the null and "other" categories are compared across populations (Table

4). Only the populations collected less than 20 km from the FEMP changed from exhibiting significant heterogeneity to being homogeneous. However, the level of significance for the 30-40 km north group decreased from  $p=0.001$  to  $p=0.04$ .

Linear regressions of frequency of the  $GPI^N$  allele with distance from the FEMP were not significant. Regressions were also performed using frequency and distance from the FEMP along each compass point; none were significant ( $p>0.05$ ).

Twenty adult males heterozygous for the null allele were maintained for use in crosses with heterozygous null females. Twenty-five of the 31 adult female treefrogs collected were gravid. Only four of the 14 crosses attempted were successful in producing viable, fertilized eggs. Two crosses (#3 and #4) represent control crosses involving either all alleles with normal activity or the null ( $GPI^N$ ) heterozygous in only one of the parents (Table 5). For cross #3 ( $GPI^{CC} \times GPI^{AC}$ ) the genotypes of offspring deviated significantly ( $p<0.05$ ) from the 1:1 expected ratio. There was no significant deviation from expected proportions (1:1) in cross #4 ( $GPI^{AN} \times GPI^{CC}$ ). In contrast, crosses involving both heterozygous males and females (#1 and #2) yielded highly significant ( $p<0.001$ ) deviations from expected ratios (Table 5). In both of the latter crosses, the  $GPI^{NN}$  genotype was expected to occur but did not appear in young of sufficient size for electrophoretic analysis; these results strongly suggest that this genotype is lethal.

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## DISCUSSION

Facemire et al. (1990) found a unique null allele at the glucose phosphate isomerase (GPI) locus in FEMP treefrog tadpoles. The allele, GPI<sup>n</sup> (their GPI<sup>c</sup>) was present in FEMP samples from both years with a frequency of approximately 20%, was expressed only in heterozygous condition and was not present in tadpoles from off-property; significant heterogeneity ( $p < 0.001$ ) existed between FEMP and off-property tadpoles at this locus.

Guttman (1990) found that the null allele occurs in a frequency approximately equal to that found on the FEMP in an area of approximately 20 km north of the FEMP (range of GPI<sup>n</sup> 15%-27%); re-examination of gel photographs from Facemire et al. (1990) determined that the GPI<sup>n</sup> had been erroneously classified as absent from their Reilly population sample. In contrast, the allele was reduced in frequency a short distance to the east and south of the FEMP. Frequencies decreased further north, southeast and south of these areas. Facemire et al. (1990) postulated that, since the GPI<sup>n</sup> allele occurred in the spring peeper tadpoles from the FEMP waste storage area but not from the reference site, their data suggested significant effects of FEMP operations on the Hyla crucifer breeding in the waste storage area. Heavy metals and radionuclides, such as those found in the waste storage area, have been shown to be mutagenic; Facemire et al. (1990) noted the necessity of additional sampling from ponds immediately around the FEMP

before the cause and site of origin for the null GPI allele could be unequivocally determined.

While the study by Guttman (1990) demonstrated that the GPI<sup>N</sup> allele has a wider distribution than anticipated by Facemire et al. (1990), the FEMP remained a possible site for its origin. Since the FEMP and surrounding industries have been active since the 1950's, sufficient time may have elapsed to account for widespread dispersal of the GPI<sup>N</sup> allele. Guttman (1990) hypothesized a potential scenario for the origin and spread of the GPI<sup>N</sup> allele but could not unequivocally determine the specific site of origin for this allele. He postulated that herbicides, pesticides, other potential mutagens or natural mutational events at any site within the area of high frequency may have been responsible for its induction. However, Guttman's (1990) data strongly suggested that the allele originated within an approximate area bounded by Reily to the north, Kirchling Road on the west and immediately south of the FEMP.

Results of analyses conducted during 1991 significantly affect the above hypotheses. It is now apparent that the GPI<sup>N</sup> allele is present in higher frequency over a much wider distribution than was previously anticipated. The northernmost site that we sampled in 1991 was at Fort Jefferson, 77 km from the FEMP; GPI<sup>N</sup> occurred at a frequency of 0.18 in that population. In 1991 we collected frogs west of the FEMP; the null allele was present in high frequency (0.20) up to 70 km west (Greensburg, IN) of the site. Previous data suggested a decrease in frequency east of the FEMP with the Bank Road sample

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(10 km off-property) exhibiting GPI<sup>n</sup> in relatively low (0.05) frequency (Guttman 1990). However, it was present in elevated frequency (0.14) in the the Mason population, 36 km east of the FEMP. Further east, at distances from 128 - 336 km from the FEMP, frequencies of GPI<sup>n</sup> ranged between 0.01 and 0.03 (sites 25-27). Dimunition of null allele frequency occurred as predicted only south of the FEMP where the cline in frequency observed earlier (0.07, Whitewater, 8 km south; 0.06, Jordan Road, 13 km south; Guttman 1990) continued to 0.01 at Rice Run, KY (site 21; 41 km south).

Chippindale (1989) conducted a study of genetic variation in spring peepers and sampled a total of 416 treefrogs from two Florida localities, Virginia, Manitoba, Nova Scotia and extensively within Ontario. He reports (Chippindale, pers. comm.) finding in two Ontario sites four tadpoles that potentially could be GPI null heterozygotes. Thirty-nine Florida frogs and 32 Virginia individuals lacked the null allele. These data, if correct, would extend the range of the null allele as far north as Ontario.

Distributional data from our studies in Ohio, Indiana and Kentucky and Chippindale's (above) demonstrate that it is not plausible to suggest that the FEMP might have been responsible for the origin of the GPI<sup>n</sup> allele. The range of the GPI<sup>n</sup> allele is too extensive to reasonably be attributed to an FEMP origin and spread since production began at the plant. The allele occurs in highest frequency in the Till Plains physiographic area (Conant 1951) of Ohio and adjacent Indiana. Only two sites

sampled (Boone County, KY; Belmont County, OH) were south of the boundary of the Pleistocene glaciers; allele frequencies appear to be highest immediately north of the boundary in southwestern Ohio and southeastern Indiana.

Although in 1991 a total of 864 field-collected Hyla crucifer were analyzed and 197 individuals were heterozygous for the GPI<sup>n</sup> allele, none of the treefrogs were homozygous for the null allele. Assuming Hardy-Weinberg equilibrium conditions and considering the populations sampled as one large unit, 11 null homozygous Hyla crucifer were expected. Combining those frogs collected in 1990 and 1991, 1647 animals were examined and the overall frequency of the null allele was 14%; a total of 462 treefrogs were heterozygous for the GPI<sup>n</sup> allele and assuming Hardy-Weinberg equilibrium conditions 32 null homozygous Hyla crucifer were expected. Jones et al. (1986) demonstrated that null heterozygotes for GPI in Clarkia, obtained after treatment of plants with the mutagen EMS, appeared to be as fit as normal homozygotes. However, null homozygotes were never recovered after selfing the null heterozygotes and were presumed to be lethal. Weeden and Wendel (1990) suggested that the presence of two, equally intense bands on an electrophoretic gel with a null heterozygote for a dimeric enzyme is a strong indication of a defective monomer; the two strands of the homozygous null dimer are unable to form an active enzyme while a null and active heterodimer can be functional. Peters and Ball (1990) obtained, after treatment of mice with the mutagen ENU, two types of null heterozygous offspring; one type produced only the active

homodimer and was identified as being heterozygous by a reduction in activity of that band. The second pattern was two-banded with both bands being equally intense; this is the appearance of the spring peeper GPI<sup>n</sup>. West et al. (1990) reported that the GPI present in normal mouse embryos is exclusively oocyte-coded until the embryonic genes are activated at 2.5-3.5 days *post coitum*. Using heterozygous null crosses, West et al (1990) demonstrated that at 7.5 days *post coitum* homozygous null embryos were present at the expected 25% frequency but by 9.5 days *post coitum* the homozygous null embryos were dead. These data, combined with the crosses in the present study support our contention from field data (Facemire et al. 1990; Guttman 1990; present study) that the homozygous null condition is lethal in spring peepers.

The data from this study strongly suggest that the FEMP was not responsible for the origin of the null GPI allele. However, this investigation has raised some extremely important basic scientific questions concerning the Hyla crucifer GPI<sup>n</sup> allele. We are uncertain of the exact timing of death of the embryos. Since we were using starch gel electrophoretic techniques to determine GPI patterns, we were unable to electrophoretically examine tadpoles until they attained adequate size. During this period many developing eggs, embryos and young tadpoles, died in each cross, especially #1 and #2 (Table 2). We are uncertain whether all frogs that died in these crosses were homozygous null. Recently we have begun to also employ cellulose acetate electrophoretic procedures; these are capable of resolving

patterns in developing eggs. Therefore, crosses of heterozygous spring peepers should be repeated. For some crosses, random samples of eggs that begin to cleave should be selected on a regular basis, sacrificed and electrophoresed to determine when embryonic genes are activated. For another set of crosses, embryos and tadpoles that die should be collected soon after death and frozen for subsequent electrophoresis to determine whether there is an equal distribution of deaths among genotypes other than the homozygous null (see cross #1, Table 2).

It is apparent from our collecting efforts that the null allele is reduced to very low frequency south of Cincinnati and within approximately 100 km east of the city. However, we do not know much about the distribution of GPI<sup>N</sup> west of the FEMP since only two sites were sampled and at 70 km west (site 23, Greensburg) it was present at a high (20%) frequency. In addition, the GPI<sup>N</sup> allele remained in high (18%) frequency 77 km north (site 24) of the plant although from Chippindale's (pers. comm.) data it is either present in very low frequency or absent from Ontario. Documentation of the distribution of the allele is imperative to elucidate both its possible site of origin and potential selection pressures responsible for its maintenance.

Selection pressures maintaining the GPI<sup>N</sup> allele must be investigated. Perhaps the best documented similar phenomenon at a single locus is the case of heterozygous advantage (overdominance for fitness) for sickle-cell hemoglobin in humans (Futuyma 1986); in this situation persons homozygous for Hb<sup>S</sup> suffer severe anemia and usually die before reproducing.

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However, in parts of Africa where falciparum malaria is prevalent the Hb<sup>AS</sup> heterozygote has a higher survival rate than the Hb<sup>A</sup> homozygote. It is critical to determine whether the Hyla crucifer GPI<sup>-n</sup> heterozygote exhibits superior fitness relative to other, normal heterozygotes.

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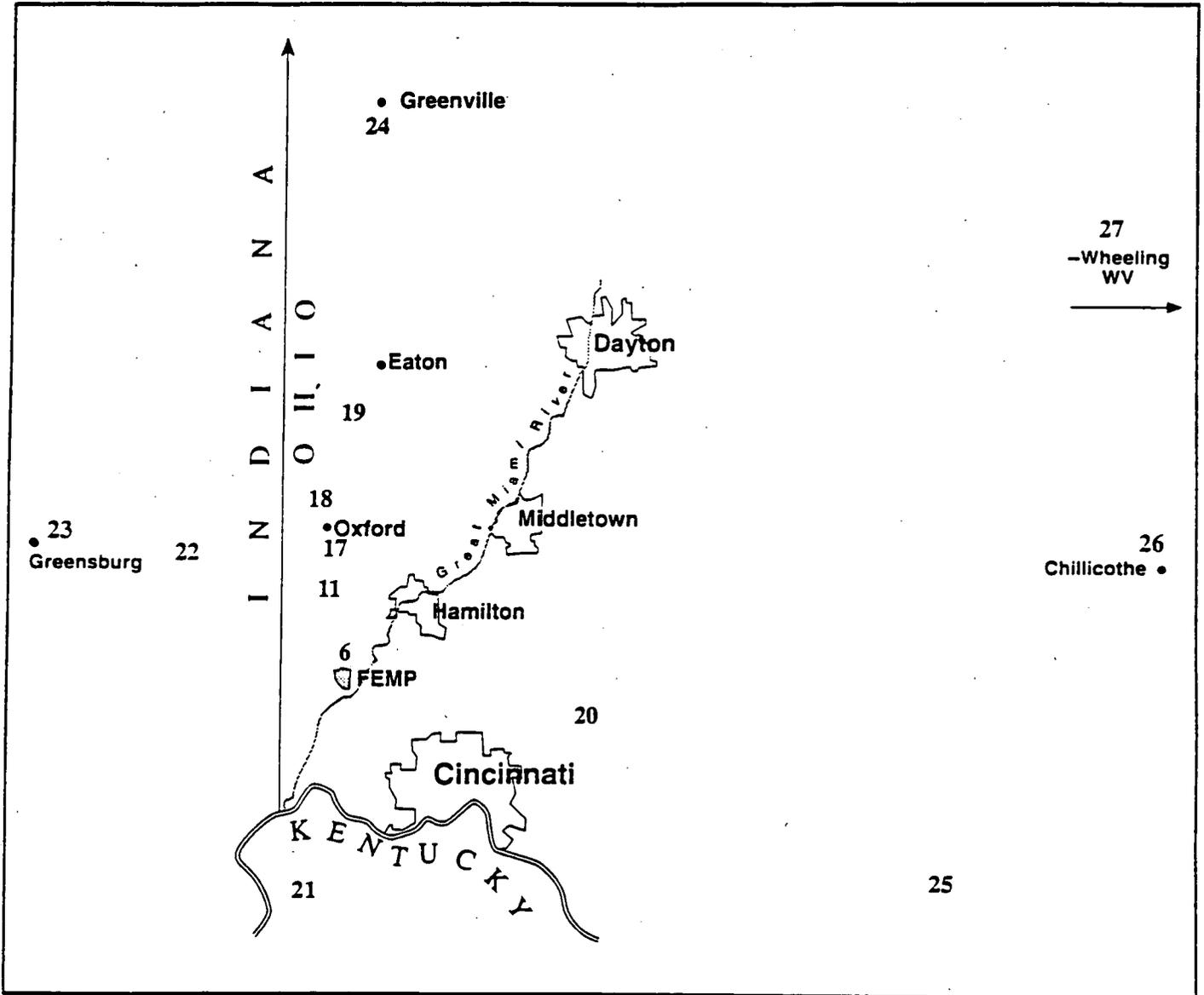


Figure 1. Location of collection sites for spring peepers *Hyla crucifer*. See Table 1 for site details.

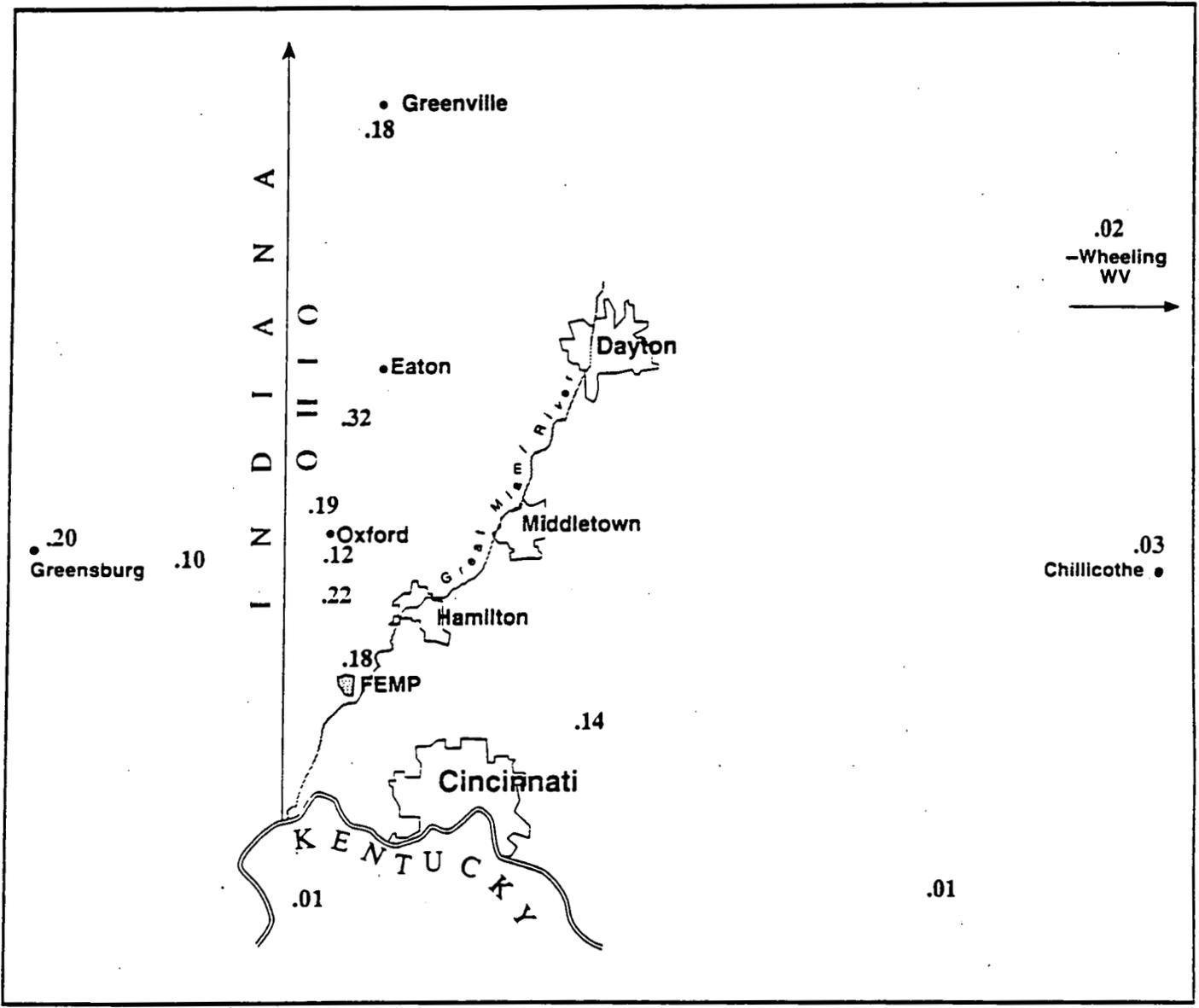


Figure 2. Allele frequencies of the GPI<sup>D</sup> allele in spring peeper *Hyla crucifer* populations sampled, Spring 1991. See Table 1 for site details.

Table 1. Collection sites for spring peeper (*Hyla crucifer*) populations examined.

Site No.	Site Abbrev.	Distance	Sample Size	County	State	Location
6	HTOP	<2 km n	75 adults	Butler	CH	Farm pond north of SR126, near Nieman Farm
11	TEET	5-10 km n	55 adults	Butler	CH	Teet's Farm, w side Chapel Rd, 1 km s jn with Jenkins Rd
17	BOOTH	15-20 km n	22 adults	Butler	CH	Pond 1km S Booth Rd, 0.7 km w US27
18	HEDGE	32 km n	42 adults	Preble	CH	Hedgerow Road
19	LAKE	43 km n	60 adults	Preble	CH	Pond adjacent to Lakengren just off SR732
20	MASON	36 km e	62 adults	Warren	CH	Ponds adjacent to Columbia Road near Mason, OH
21	RICE	41 km s	49 adults	Boone	KY	Rice Run Rd., 8 km w I71
22	MARY	38 km w	44 adults	Franklin	IN	On IN101, 4.2 km n of I74
23	GREEN	70 km w	119 tadpoles	Decatur	IN	3 km s Knorr Corner, on US421 s Greensburg
24	FORT	77 km n	42 adults	Darke	CH	w of US127, s Fort Jefferson
25	BLUE	128 km e	118 tadpoles	Adams	CH	flooded field adjacent to OH125 in Blue Creek
26	CHILL	142 km e	124 tadpoles	Ross	CH	Lunbeck Rd., 1 km s jn OH104
27	BELL	336 km e	52 adults	Belmont	CH	Blaine, adjacent to Blaine Cemetery

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Table 2. Allele frequencies at the glucose-6-phosphate isomerase (GPI) locus in populations of treefrogs (*Hyla crucifer*). Site abbreviations and numbers refer to Table 1. N indicates number of individuals sampled from each site.

NAME	<u>POPULATION</u>												
	HTOP	TEET	BOOTH	HEDGE	LAKE	MASON	RICE	MARY	GREEN	FORT	BLUE	CHILL	BELL
POP. #	6	11	17	18	19	20	21	22	23	24	25	26	27
(N)	75	55	22	42	60	62	49	44	119	42	118	124	52
Allele													
a	0.17	0.13	0.05	0.14	0.03	0.30	0.14	0.10	0.10	0.10	0.27	0.29	0.24
b	0.04				0.05	0.25		0.06	0.03	0.07	0.02	0.01	
c	0.62	0.75	0.86	0.67	0.60	0.30	0.85	0.74	0.67	0.65	0.70	0.67	0.74
e						0.01							
n	0.17	0.12	0.09	0.19	0.32	0.14	0.01	0.10	0.20	0.18	0.01	0.03	0.02

Table 3. Contingency chi-square analyses for genetic heterogeneity at the GPI locus among treefrog populations. All alleles were considered. Comparisons are within distance classes. Where sufficient populations were sampled from different compass points within a distance class, comparisons were made by compass direction. \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , NS = not significant.

0-20 km n	30-40 km	30-40 km n	$\geq 70$ km	$\geq 70$ km e
..	...	...	...	NS

Table 4. Contingency chi-square analyses for genetic heterogeneity at the GPI locus among treefrog populations. Two classes of alleles were considered: null and other. Comparisons are within distance classes. Where sufficient populations were sampled from different compass points within a distance class, comparisons were made by compass direction. \* $p \leq 0.05$ , \*\*\* $p \leq 0.001$ , NS = not significant.

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0-20 km n	30-40 km	30-40 km n	$\geq 70$ km	$\geq 70$ km e
NS	...	.	...	NS

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Table 5. Crosses made to determine mode of inheritance and potential lethality of null allele. The chi-square test (corrected for small samples) was used to determine significance of observed compared with expected genotypic values.

Cross #	Female #, site, genotype	x	Male #, site, genotype	# analyzed	Number of observed (expected) genotypes				p
					ac	an	cn	nn	
1	13 (pop. 11) an	x	17 (pop. 11) cn	38	29 (9.5)	8 (9.5)	1 (9.5)	0 (9.5)	<0.001
2	12 (pop. 6) cn	x	22 (pop. 6) cn	55	25 (13.8)	30 (27.5)	0 (13.8)		<0.001
3	1 (pop. 21) cc	x	48 (pop. 27) ac	108	43 (54)	65 (54)			< 0.05
4	1 (pop. 6) an	x	5 (pop. 6) cc	85	41 (42.5)	44 (42.5)			n.s.

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