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CHARACTERIZATION OF THE TREEFROG NULL ALLELE - (USED AS A REFERENCE IN OU 5 RI REPORT)

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CHARACTERIZATION OF THE TREEFROG NULL ALLELE

by

Sheldon I. Guttman

Department of Zoology
Miami University
Oxford, Ohio 45056

December 1990

Prepared for the
FEED MATERIALS PRODUCTION CENTER
Westinghouse Materials Company of Ohio
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Approved: Sheldon I. Guttman
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3

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4

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TABLE OF CONTENTS

Page

Introduction.....1

Methods.....3

Results.....4

Discussion.....7

Acknowledgments.....11

Literature Cited.....12

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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 Feed Materials Production Center (FMPC) populations using electrophoretic techniques. These data are being used as an indicator of stress by comparing populations on and off the FMPC site.

Anurans (frogs and toads) are ideal organisms for use as monitors of environmental stress. 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 stay around their natal pond or disperse up to 10 km (Ewert 1969). However, 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 FMPC and because they breed onsite. 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).

Spring peeper tadpoles (N=80) were collected 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 offsite 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 FMPC treefrog tadpoles. GPI is an essential glycolytic enzyme. The allele, GPI^C, is a null (inactive under assay conditions). It was present in FMPC samples with a frequency of approximately 20% and was expressed only in heterozygous condition. This allele was not present in tadpoles from offsite and significant heterogeneity ($p < 0.001$) existed between FMPC and offsite tadpoles at this locus. Guttman (1985) recently reviewed the literature on biochemical population genetics of frogs and toads and did not find 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 been demonstrated to be lethal. Burkhart et al. (1984) analyzed null alleles in *Drosophila* and noted that GPI was the only essential locus among

7

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).

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 effect occurs relative to the frequency of the null allele offsite and to determine the origination site of the null allele.

METHODS

Three hundred and seventy-four adult *Hyla crucifer* were collected from 15 sites including the FMPC (Sites 1-15; Table 1, Figure 1); all but three of the adults were males. 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. Males ceased calling after 14 May 1990; therefore, additional adults could not be collected. Sample sizes were increased at individual sites by returning to those sites and collecting *Hyla crucifer* tadpoles. In addition, two new sites (16 and 17; Table 1, Figure 1) were sampled using only tadpoles. A total of 419 *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 -70° C 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 (Sigma Chemical Co., St.Louis) gel, 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). Prior to commencing the study, protocols were submitted and approved by WMCO.

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

Twenty adult males heterozygous for the null allele were maintained for use in crosses with heterozygous null females. The three adult female treefrogs collected were gravid.

However, analysis of their GPI patterns indicated that they lacked the null allele and could not be used for crosses.

A total of five GPI alleles were detected in the 783 *Hyla crucifer* examined. Two represent alleles not observed by Facemire et al. (1990). The additional alleles necessitated a change in allelic nomenclature; alleles GPI^b and GPI^c of Facemire et al. (1990) were designated GPI^c and GPI^n, respectively. The two new alleles were labeled GPI^b and GPI^d. Allele frequencies in the populations studied are listed in Table 2; the distribution of the null allele GPI^n is mapped in Fig. 2. No individual sampled was homozygous for GPI^n.

Highest frequencies of GPI^n occur in the immediate vicinity of the FMPC, although the Teet's Farm and Riley population frequencies approximate that of the FMPC vicinity. Allele frequencies for GPI^n decrease dramatically south and east of the FMPC, attaining frequencies between six and eight percent between 5-10 km from the FMPC. In contrast, allele frequencies for GPI^n remain high north of the FMPC over at least 15 km; they appear to decrease at a distance of approximately 20 km (12%; population 17).

Two types of contingency chi-square analyses were performed. The first considered all five alleles in the populations sampled; the second analysis pooled alleles A-D into an "other" category and compared the frequencies of the null and "other" categories across populations.

Using all alleles, frequencies for GPI alleles in tadpoles and adults from the FMPC are not significantly different (Table

3); in addition, those samples collected from the vicinity of the FMPC are not significantly different from each other nor from FMPC treefrogs. However, those populations within a 5-10 km radius of the FMPC are significantly heterogeneous ($p < 0.00000$); the majority of the contribution to the high chi-square value derives from the high frequency of GPI^a in the Whitewater sample, low frequency of GPI^b in the Teet's Farm sample, presence of GPI^d in the Wieks Road frogs and uneven distribution of GPI^n. Separation of the populations into northern and southern groups resulted in the maintenance of within-group heterogeneity (Table 3). Between 10-15 km, the two populations sampled are significantly heterogeneous ($p<0.025$) due to the high frequency of GPI^n in the northern Garner Road sample compared to the treefrogs from Jordan Road located south of the FMPC. Those populations sampled from greater than 15 km were all from north of the FMPC. Significant heterogeneity ($p<0.0006$) was primarily due to the unequal distribution of three (GPI^a, GPI^d, GPI^n) of the five alleles. Comparison of only the Riley and Garver Road populations, removing population 17 from the comparison, yielded data indicating the former two populations are homogeneous at GPI.

Results differ markedly when alleles A-D are pooled into an "other" category and the frequencies of the null and "other" categories are compared across populations (Table 4). While the populations between 5-10 km from the FMPC continue to exhibit significant heterogeneity, analysis of these samples by direction relative to the plant indicates homogeneity within

both the northern and southern groupings. In addition, heterogeneity between the Jordan and Garner Road samples is accentuated. Finally, those populations 15-20 km from the FMPC (15-17) become marginally homogeneous (p=0.08, Table 4).

DISCUSSION

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

The present study was initiated to determine whether the frequency of the GPI^n allele decreases in *Hyla crucifer* with increasing distance from the FMPC. Since Facemire et al. (1990) did not find this allele in their reference sample, located approximately 17 km from the FMPC, the outer perimeter for sampling was set at a distance of 10 km from the plant. Our data indicated that the null allele occurs in a frequency approximately equal to that found on the FMPC 5-10 km north of the plant (22%, pop. 11, Teet's Farm). However, the allele becomes greatly reduced in frequency an equivalent distance to the east (5%, pop. 13, Bank Road) and south (7%, pop. 9, Whitewater). We, therefore, extended our collecting area and managed to sample within a 20 km radius of the FMPC before the

spring peeper breeding season ended and all tadpoles metamorphosed from known breeding ponds.

The presence of a significant frequency of GPI^n in population 15 (15%, Garver Rd) induced us to resample the Reily (Indian Creek County Park) population used as a reference by Facemire et al. (1990), since the latter population is south of the Garver Road collection site. Analysis of 89 spring peeper tadpoles from site 16 demonstrated that GPI^n was present in high frequency (21%) in that population as well. Subsequent reexamination of photographs of gels obtained by Facemire et al. (1990) documented that GPI^n was indeed present in their 1987 sample from site 16 but had been misidentified.

Data obtained in the present study indicate an area of approximately 20 km (including populations 1-7, 10-12, and 15-16) where the frequency of GPI^n ranges between 15%-27%. Frequencies decrease north, southeast and south of this area. Facemire et al. (1990) postulated that, since the GPI^n allele occurred in the spring peeper tadpoles from the FMPC waste storage area but not from the reference site, their data suggested significant effects of FMPC 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 FMPC before the cause and site of origin for the null GPI allele could be unequivocally determined.

While the present study demonstrates that the GPI^n allele has a wider distribution than anticipated by Facemire et al. (1990), the FMPC remains a possible site for its origin. The FMPC and surrounding industries have been active since the 1950's. Therefore, sufficient time may have elapsed to account for widespread dispersal of the GPI^n allele. Platz et al. (1990) demonstrated that frogs (Rana berlandieri), after introduction by humans, dispersed at a rate of approximately 12 km per year along the Colorado and Gila Rivers in California and Arizona. While the movement of the Rana occurred along the banks of, or in, the rivers, Hyla crucifer migrate over land following metamorphosis and are found as adults in wooded areas. Land immediately north of the FMPC is flat and has been extensively cleared for agriculture; wooded habitat is scarce. Dispersing frogs, moving during wet periods, would have to migrate great distances before reaching habitat suitable for existence as adults. In contrast, extensive hilly, wooded areas to the south and east of the FMPC would tend to reduce dispersal distances. Additionally, increasing urbanization in these directions would make successful dispersal of the treefrogs more difficult.

While the above scenario for the origin and spread of the GPI^n allele is attractive, distributional data obtained during this study indicate that the specific site of origin for this allele cannot be unequivocally determined. 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, the marked decrease in frequency of the allele in populations 8-9, 13-14 and 17 strongly suggest that the allele originated within an approximate area bounded by Reily to the north, Kirchling Road on the west and immediately south of the FMPC.

Although 783 Hyla crucifer were analyzed and 266 individuals were heterozygous for the GPI^n 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, 22 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. Their data, combined with those reported by Burkhart et al. (1984) for Drosophila and Ravindranth et al. (1987) for humans, support the contention by Facemire et al. (1990) that the homozygous null condition is lethal in spring peepers.

Unfortunately, authorization for this study was issued near the end of Hyla crucifer's breeding season and only three gravid

females, all lacking the null allele, could be collected. Crosses of heterozygous spring peepers should be made next spring to unequivocally demonstrate whether the GPI^n allele, when homozygous, is lethal during early development.

In addition, sampling west of the FMPC as well as at distances greater than 20 km, along the remaining compass points, must be conducted to confirm whether the frequency of this unique null allele continues to decline with increasing distance from the FMPC and vicinity.

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Appreciation is extended to Linda England and Brinley Varchol, Environmental Monitoring, WMCO, for their cooperation and enthusiastic support of this project. I am also extremely grateful to Michael Wright for his extraordinary talent and perseverance in collecting treefrogs. Marc Albrecht and Scott McClain also assisted in the collecting efforts. Elisa Ruschman and Debbie Dean were primarily responsible for orchestrating the electrophoretic analyses. The help of Ian Bell in generating computer maps was invaluable.

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

Site No.	Site Abbrev.	Sample Size	County	Location
1	FMPCA	9 adults	Hamilton	Meteorological tower, near fly ash pile and north leased grazing area
2	FMPCT	88 tadpoles	Hamilton	Meteorological tower
3	POND1	44 adults	Hamilton	Paddy's Run Road, w of Boundary Station 6
4	RR	16 adults	Butler	Paddy's Run Road, at railroad crossing, in a ditch by pallet company
5	NIEM	8 adults	Butler	Nieman Farm
6	HTOP	22 adults	Butler	Farm pond north of SR126, near Nieman Farm
7	BRUSH	21 adults	Hamilton	Farm pond on SR128 near jn with Hanover Road
8	WIEK	31 adults	Hamilton	Jn Wiek & Dry Ridge Rds
9	WHITE	41 adults	Hamilton	Ditch along SR128, 1.2 km n of Miamitown
10	KIRCH	17 adults 46 tadpoles	Butler	S side Kirchling Rd, 0.7 km w of jn with US27
11	TEET	11 adults 100 tadpoles	Butler	Teet's Farm, w side Chapel Rd, 1 km s jn with Jenkins Rd
12	GARN	42 adults	Butler	Garner Rd across from Bunker Hill Boy's Center
13	BANK	43 adults	Hamilton	Bank Rd nr Hughes Rd
14	JORD	42 adults	Hamilton	S side of Jordan Rd, 2.1km w of Zion Rd
15	GARV	41 adults	Butler	Garver Rd 0.5 km n Elliot Rd
16	REILY	89 tadpoles	Butler	Pond on w side Indian Creek Park
17	BOOTH	77 tadpoles	Butler	Pond 1km S Booth Rd, 0.7 km w US27

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.

	POPULATION																
NAME	FMPCA	FMPCT	POND1	RR	NIEM	HTOP	BRUSH	WIEK	WHITE	KIRCH	TEET	GARN	BANK	JORD	GARV	REILY	BOOTH
POP. #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
(N)	9	88	44	16	8	22	21	31	41	63	111	42	43	42	41	89	77
Allele																	
a	0.06	0.19	0.16	0.06	0.13	0.07	0.10	0.08	0.40	0.11	0.12	0.11	0.23	0.18	0.06	0.10	0.01
b	0.00	0.05	0.05	0.03	0.12	0.05	0.05	0.10	0.10	0.12	0.01	0.00	0.07	0.02	0.01	0.00	0.01
c	0.72	0.54	0.52	0.69	0.56	0.71	0.67	0.73	0.43	0.59	0.66	0.70	0.64	0.74	0.77	0.69	0.87
d	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
n	0.22	0.22	0.27	0.22	0.19	0.18	0.18	0.08	0.07	0.18	0.22	0.19	0.06	0.06	0.15	0.21	0.12

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 < 0.05, **p < 0.01, ***p < 0.001, NS = not significant.

FMPC	<2 km	5-10 km	5-10 km n	5-10 km s	10-15 km	15-20 km	15-17 km
NS	NS	***	***	**	*	***	NS

6385

23

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 < 0.01, ***p < 0.001, NS = not significant, values between p = 0.06 and p = 0.09 are listed.

FMPC	<2 km	5-10 km	5-10 km n	5-10 km s	10-15 km	15-20 km	15-17 km
NS	NS	***	NS	NS	**	0.08	NS

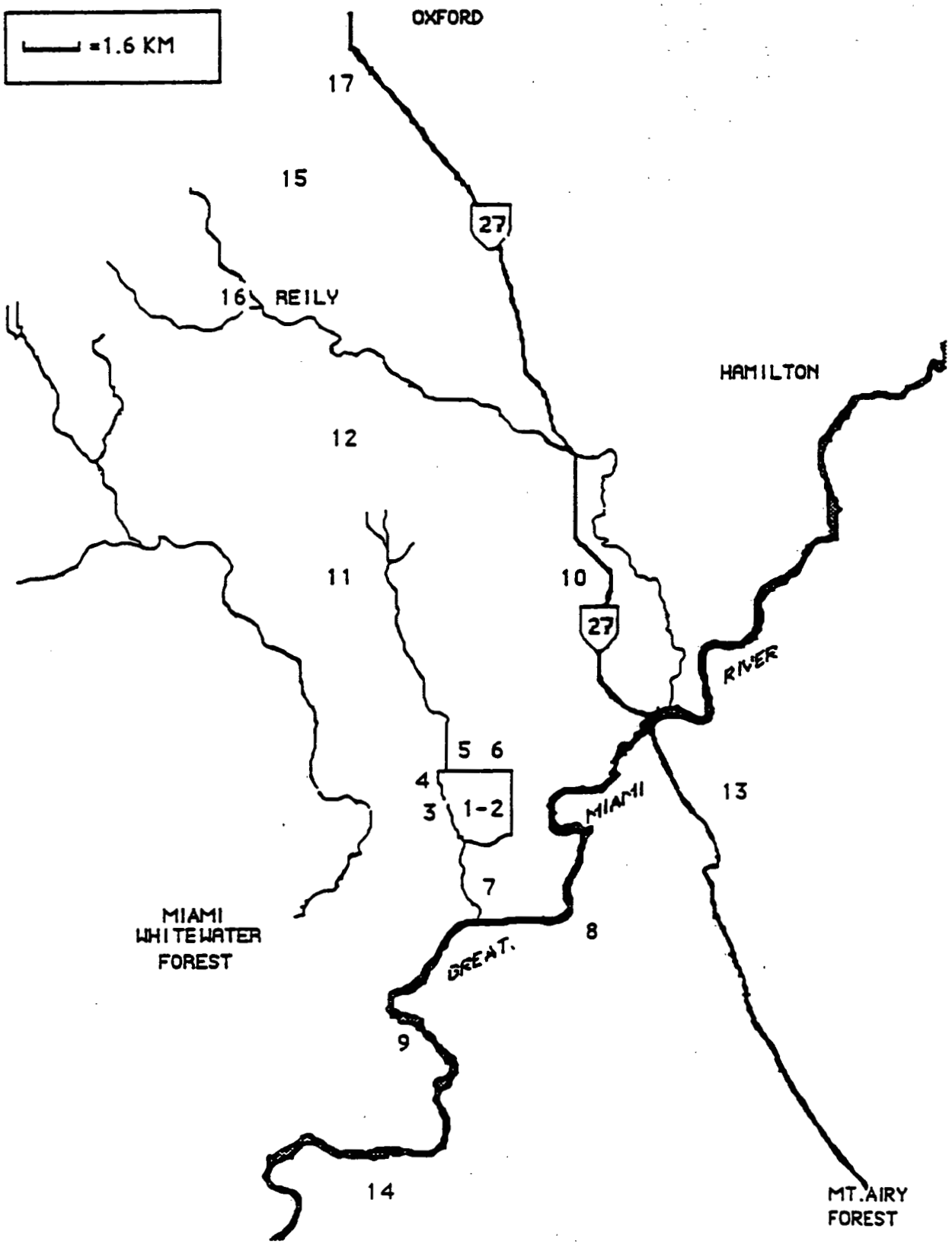

Figure 1. Location of collection sites for spring peepers Hyla crucifer. See Table 1 for site details.

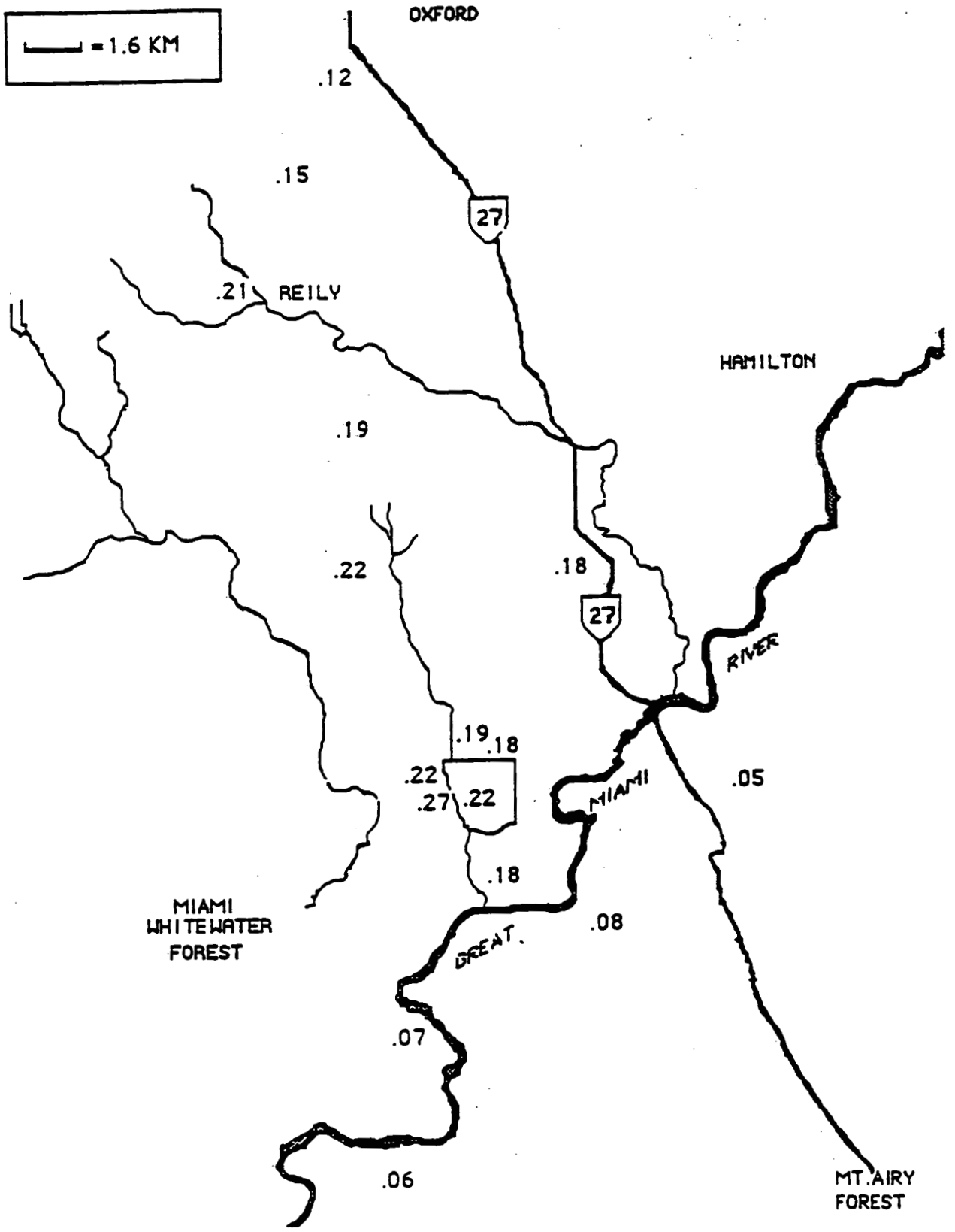

Figure 2. Allele frequencies of the GPI^n allele in spring peeper Hyla crucifer populations sampled, Spring 1990. See Table 1 for site details.