# Biochemical Genetic Variation in the Family Simuliidae: Electrophoretic Identification of the Human Biter in the Isomorphic *Simulium jenningsi* Group[1]

BERNIE MAY,[2,3] LEAH S. BAUER,[2] ROBERT L. VADAS,[2] AND JEFFREY GRANETT[2]

ABSTRACT

This paper describes inter- and intraspecific protein variation in the 3 closely related species of the *Simulium jenningsi* black fly group, *S. jenningsi* Malloch, *S. nyssa* Stone and Snoddy, and *S.* n. sp. P. Snoddy and Bauer. Variation is described at single loci coding for the enzymes, phosphoglucomutase, phosphoglucoisomerase, malate dehydrogenase, and lactate dehydrogenase. Data are analyzed to assign genetic relationships among the 3 species and to suggest their status as sibling species. The application of electrophoretic methodology has revealed *S.* n. sp. P. to be the principle biter of humans of this group during the early autumn in north-central Maine.

Black flies of the *Simulium* subgenus *Phosterodoros* are morphologically homogeneous (Stone and Snoddy 1969). Three species within this subgenus, *S. jenningsi* (Malloch 1914), *S. nyssa* (Stone and Snoddy 1969), and *S.* n. sp. P. (Snoddy and Bauer 1977), are isomorphic in the adult stage, although identifiable in the larval and pupal stages. We group these three species as the *S. jenningsi* group, based on their isomorphic nature in the adult stage.

The *S. jenningsi* group is responsible for autumn human biting activity in north-central Maine[4]. The similarity of its adults has prevented a determination of the species actually responsible for the biting. It is critical to determine which species are involved in the biting for a rational approach to black fly control.

Townson (1976) described an electrophoretic approach to separate members of the *S. damnosum* group, although his findings were not definitive. We describe the use of this technique to differentiate members of the isomorphic *S. jenningsi* group.

## METHODS AND MATERIALS

Pupae from 10 sites within the Penobscot River watershed were identified to species. The pupae were held until emergence at 21°C in individual 7-dram vials on moist cotton. Adult biters were collected in the act of biting and brought to the laboratory in individual 12×75-mm culture tubes. The newly emerged adults of known species and the biters were either immediately electrophoresed or frozen for up to 2 wk at −60°C.

Individual black flies were homogenized with a glass rod with 1–2 drops of 0.05 M TrisHCl (pH 7.1) in 12×75-mm glass culture tubes. The homogenates were subjected to horizontal starch gel electrophoresis (Electrostarch)[5].

Staining followed the general recipes of Brewer (1970) and Shaw and Prasad (1970). Components were routinely estimated visually as quantities were not found to be critical for obtaining reliable staining.

Allelic nomenclature followed that of Richmond (1972). Table 1 lists the 11 enzymes for which activity was detected, their abbreviations, and the appropriate buffer systems. When italicized, these abbreviations represent the locus coding for the enzyme. Allelic variants are designated by the relative electrophoretic mobility of the homomeric band for which they code. One allele, usually the most common, is designated 100 and the other alleles are then assigned a number reflecting their homomer's relative mobility to the homomer for allele 100. For example, a variant allele for a dimeric enzyme (XYZ) which produces a homodimer which migrates half as far as the homodimer produced by allele 100 would be designated *XYZ* (50).

## RESULTS AND DISCUSSION

The small size of the black flies and the generally weak activity and monomorphic nature for most enzymes resulted in the use of only 4 enzymes: PGM, PGI, MDH, and LDH. Each of the 4 enzymes appeared to be coded by a single locus, based on the symmetric banding pattern of heterozygous individuals and conformation of electrophoretic phenotypes to the expected Hardy-Weinberg proportions. PGI and MDH were dimeric and PGM was monomeric. The compact nature of the banding pattern for LDH heterozygotes precluded a determination of whether this enzyme was tetrameric as has been found in all other organisms. The observed electrophoretic banding patterns are diagrammed in Fig. 1 with the presumed genotypic basis given for each electrophoretic phenotype.

Allele frequencies for each locus are presented in Table 2. Small sample sizes prevented an accurate estimation of allele frequencies for each site. Chi-square tests for fit to Hardy-Weinberg proportions revealed only the distribution for PGM phenotypes in *S. nyssa* to be significantly out of Hardy-Weinberg. These data suggest there is probably little populational differentiation within the Penobscot watershed for each of these 3 species. This finding is not surprising, owing to the impressive dispersing abilities

[1] Received for publication April 8, 1977.

[2] Dept. of Botany and Plant Pathology and Dept. of Entomology, Univ. of Maine, Orono 04473.

[3] Present address: Dept. of Biology, Pennsylvania State Univ., University Park 16802.

[4] L. H. Waters. 1969. Bionomics of black flies in Maine. M.S. Thesis. University of Maine, Orono.

[5] B. May. 1975. Electrophoretic variation in the genus *Oncorhynchus*: The methodology, genetic basis, and practical applications to fisheries research and management. M.S. Thesis. University of Wash., Seattle. 95 pp. University Microfilms #13-10, 112.

Table 1.—Enzymes and buffer systems used in this study.

| Enzyme | Abbreviation | Buffer System |
|---|---|---|
| Acid Phosphatase | ACP | 1 |
| Diaphorase | DIA | 1 |
| Esterase | EST | 1 |
| α-Glycerophosphate dehydrogenase | AGP | 2 |
| Hexokinase | HK | 1 |
| Isocitrate dehydrogenase | IDH | 2 |
| Lactate dehydrogenase | LDH | 2 |
| Malate dehydrogenase | MDH | 2 |
| Phosphoglucoisomerase | PGI | 1,2 |
| Phosphoglucomutase | PGM | 1 |
| 6-Phosphogluconate dehydrogenase | 6PGDH | 2 |

Buffer System

1—Described by Ridgway, Sherburne, and Lewis (1970)

*Gel buffer* (pH 8.5)
Tris base (0.03m) Lithium hydroxide (0.0006m)
Citric acid (0.005m) Boric acid (0.003m)

*Electrode buffer*
Lithium hydroxide (0.06m) Boric acid (0.3m)

2—Described by Clayton and Tretiak (1972)

*Electrode buffer* (pH 6.1)
Citric acid (0.04m)
pH adjusted with N-(3-Aminopropyl)-morpholine

*Gel buffer*
use electrode buffer diluted 1:10.

of black fly larvae and adults (Fredeen 1956, Rubtsov 1964, and Baldwin 1975).

The allele frequencies in Table 2 yield direct insight into the central question regarding which member(s) of the *S. jenningsi* group are responsible for the human biting activity. *S. jenningsi* can be ruled out as a significant biter since it is fixed for *MDH*(*77*) while the biters have a frequency of 0.01 for this allele. An examination of *LDH* substantiates this conclusion. *S.* n. sp. P. and the biters have allele frequencies of 0.92 and 0.88, respectively, for *LDH*(100) while *S. nyssa* has a frequency of 0.09 for this allele. We therefore conclude that *S.* n. sp. P. is the principle autumn biter in the Penobscot River watershed of Maine.

It is possible that *S. nyssa* is making a minor contribution to the biting population based on the *LDH* data. Using the *LDH*(100) frequencies, we calculate a proportional contribution by *S. nyssa* as follows:

$$F(B) = P(N)F(B) + (1-P(N))F(P)$$

$$\text{or} \quad P(N) = \frac{F(B)-F(P)}{F(N)-F(P)} = \frac{.88-.92}{.09-.92} = 0.05$$

where P(N) = proportion of *S. nyssa* in biting population
F(B) = frequency of *LDH*(100) in biters
F(P) = frequency of *LDH*(100) in *S.* n. sp. P.
F(N) = frequency of *LDH*(100) in *S. nyssa*

This does not imply that *S. nyssa* constitutes 5% of the biting population because there is not absolute species specificity at any of these loci and it is therefore impossible to unambiguously assign an individual biter to one species or another.

The allele frequency variances in Table 2 for *LDH* can be used to provide a range of error around our estimate of 5% for *S. nyssa*. Using 2 SE to bring the allele frequencies for *S. nyssa* and the biters as close together and *S.* n. sp. P. as far away as possible we see that:

$$P(N) = \frac{F(B)-F(P)}{F(N)-F(P)} = \frac{.83-.99}{.14-.99} = 0.19$$

The reciprocal gives *S. nyssa* a zero-contribution to the biting population. We conclude that *S.* n. sp. P. is the principle biter with *S. nyssa* making a possible contribution of 0–19% with a best estimate of 5%.

Table 2.—Allele frequencies at the 4 loci coding for the enzymes *PGI, PGM, MDH,* and *LDH* in black flies of the *Simulium jenningsi* group (N = sample size).

| | *PGI* | | | | | | | | | *PGM* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | N | 13 | 58 | 70 | 100 | 134 | 156 | 2 s.e. | $\chi^2$ | N | 90 | 100 | 110 | 2 s.e. | $\chi^2$ |
| *S.* n. sp. P. | 41 | | .17 | | .82 | .01 | | ±.08 | 4.12 | 6 | .17 | .50 | .33 | ±.29 | 7.77 |
| *S. nyssa* | 106 | | .04 | .01 | .83 | .13 | | ±.05 | 4.39 | 42 | .10 | .74 | .17 | ±.10 | 10.20 |
| *S. jenningsi* | 34 | | .03 | | .85 | .10 | .02 | ±.09 | 1.49 | 18 | | .86 | .14 | ±.11 | 2.06 |
| biters | 79 | .01 | .20 | .02 | .75 | .03 | | ±.07 | 8.50 | 32 | .14 | .75 | .11 | ±.11 | 0.88 |

| | *MDH* | | | | | | | *LDH* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | N | 77 | 88 | 100 | 110 | 2 s.e. | $\chi^2$ | N | 83 | 100 | 113 | 2 s.e. | $\chi^2$ |
| *S.* n. sp. P. | 49 | | .05 | .95 | | ±.04 | 0.12 | 32 | .02 | .92 | .06 | ±.07 | 0.21 |
| *S. nyssa* | 82 | .01 | | .99 | .01 | ±.02 | 0.00 | 57 | | .09 | .91 | ±.05 | 0.49 |
| *S. jenningsi* | 31 | 1.00 | | | | | 0.00 | 30 | | .03 | .97 | ±.04 | 0.04 |
| biters | 119 | .01 | | .99 | | ±.01 | 2.01 | 81 | | .88 | .12 | ±.05 | 0.92 |

* s.e. = $\sqrt{\frac{P(1-P)}{2N}}$ where P = frequency of allele 100.

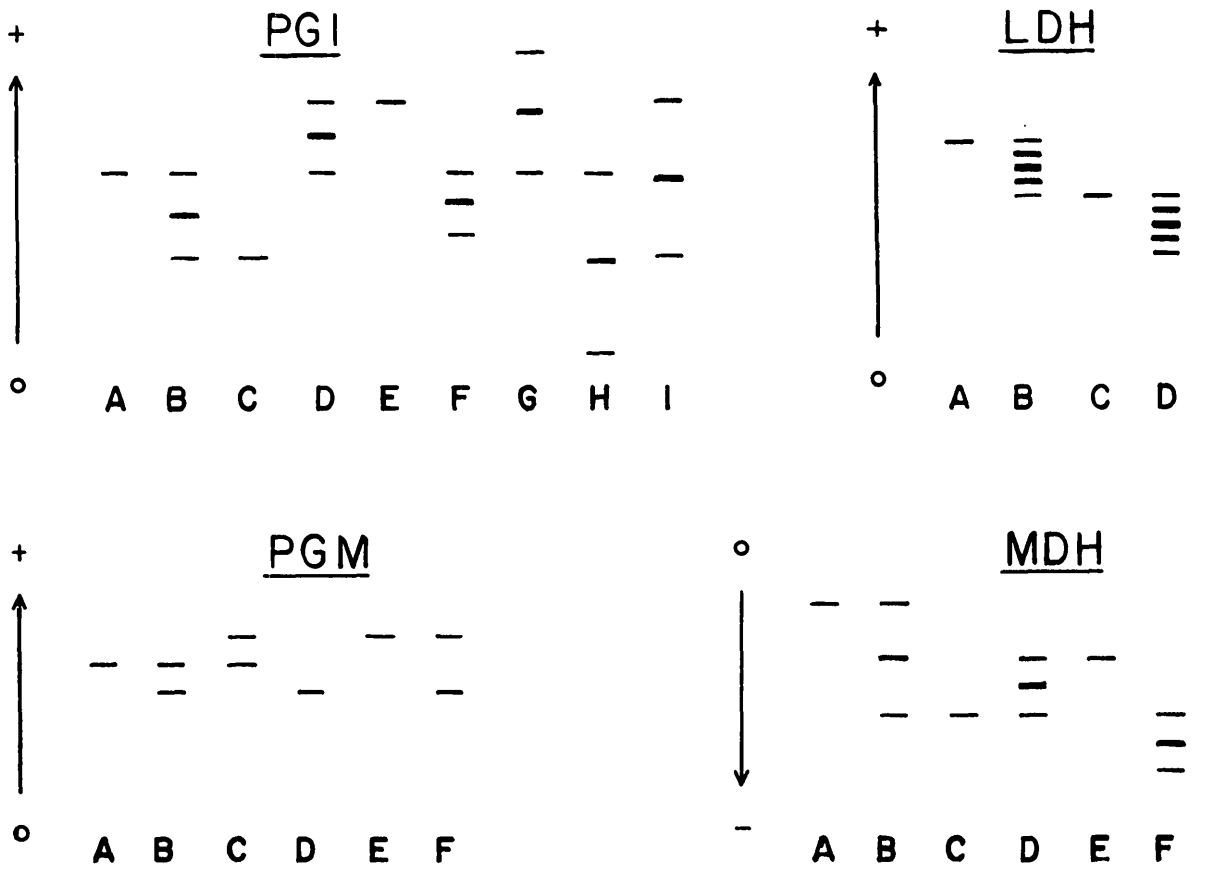


FIG. 1.—Observed electrophoretic banding patterns with assumed genotypic bases. *PGI:* A-(100/100), B-(58/100), C-(58/58), D-(100/134), E-(134:134), F-(70/100), G-(100/156), H-(13/100), and I-(58/134). *LDH:* A-(113/113), B-(100/113), C-(100/100), and D-(83/100). *LDH* heterozygotes are diagrammed as tetrameric although the exact number of bands could not be determined. *PGM:* A-(100/100), B-(90/100), C-(100/110), D-(90/90), E-(110/110), and F-(90/110). *MDH:* A-(77/77), B-(77/100), C-(100/100), D-(88/100), E-(88/88), and F-(100:110).

No exact quantitative data exist on the stream distribution of immatures of these 3 species. However, the observed distribution of immatures suggested that *S. jenningsi* and *S. nyssa* both occurred in higher numbers than *S.* n. sp. P. *S. jenningsi* tended to occur in more restricted habitats with only slight overlap with the other 2 species. *S. nyssa* was usually found with *S.* n. sp. P. in varying ratios.

Davies and Peterson (1956) have suggested, based on reduced mouth parts, that the females of some species of black flies do not require a blood meal before ovipositing (autogenous). The host specificity of black fly species requiring a blood meal (anautogenous) has been well documented (Anderson and Defoliart 1961, Lowther and Wood 1964, Moore and Noblet 1974). Our data suggest that *S. nyssa* and *S. jenningsi* females are either autogenous or that they are using a non-human host for their blood source. Further studies should resolve this question.

Morphological criteria have historically been the only means of studying the evolutionary divergence in existing species. Ayala (1975) has used electrophoretic data coupled with morphological evidence to determine the evolutionary state of various groups of *Drosophila* species: local populations, subspecies, semispecies, sibling species, or morphologically distinct species. Based on his criteria, we suggest that these 3 species of the *S. jenningsi* group are sibling species. They are morphologically very similar (isomorphic in the adult stage). Secondly, at each of the 4 loci considered, the 3 species are either very similar in allele frequencies or nearly fixed for alternate alleles.

Genetic similarities and distances were calculated for these 3 species of the *S. jenningsi* group and for the biters using the formulae of Rogers (1972). These coefficients which measure the mean similarity between allele frequency vectors over the range of loci are presented in Table 3. The use of these 4 highly polymorphic loci with the exclusion of less polymorphic and monomorphic loci prevent these data from being used to compare the differentiation in this group with that found in other groups of sibling species (Ayala 1975). Inclusion of these other loci would change the absolute values of genetic

Table 3.—Genetic similarity and genetic distance for black flies of the *Simulium jenningsi* group on the basis of gene frequencies at the loci coding for *PGI, PGM, MDH,* and *LDH.*

| | Genetic similarity | | | |
|---|---|---|---|---|
| | P | J | N | B |
| P | | .42 | .69 | .90 |
| J | .58 | | .70 | .47 |
| N | .31 | .30 | | .75 |
| B | .10 | .53 | .25 | |
| Genetic distance | | | | |

P = *S.* n. sp. P.; J = *S. jenningsi;* N = *S. nyssa;* B = biters.

similarities but not the relative differences between them. *S.* n. sp. P. and the biters have a similarity of almost unity (S = 0.90) supporting our conclusion that *S.* n. sp. P. is the principle human biter. Among these 3 species we note that *S.* n. sp. P. and *S. jenningsi* are more closely related to *S. nyssa* (S = 0.69 and S = 0.70, respectively) than *S.* n. sp. P. and *S jenningsi* are to one another (S = 0.42).

ACKNOWLEDGMENT

We thank Peter S. Gagnon and David E. Leonard for critical reviews of this manuscript and Edward R. Bauer for assistance in field sampling. Portions of this research were supported by C.S.R.S. grant #616-15-94 and by Hatch Project #295.

REFERENCES CITED

**Anderson, J. R., and G. R. Defoliart.** 1961. Feeding behaviour and host preferences of some black flies (Diptera: Simuliidae) in Wisconsin. Ann. Entomol. Soc. Am. 54: 716–29.

**Ayala, F. J.** 1975. Genetic differentiation during the speciation process. Evol. Biol. 8: 1–78.

**Baldwin, W. F.** 1975. Dispersal pattern of black flies (Diptera: Simuliidae) tagged with $^{32}P$. Can. Entomol. 107: 113–8.

**Brewer, G. J.** 1970. An Introduction to Isozyme Techniques. Academic Press, New York.

**Clayton, J. W., and D. N. Tretiak.** 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish. Res. Bd. Can. 29: 1169–72.

**Davies, D. M., and B. V. Peterson.** 1956. Observations on the mating, feeding, ovarian development, and oviposition of adult black flies (Simuliidae, Diptera). Can. J. Zool. 34: 615–55.

**Fredeen, F. J. H.** 1956. Black flies (Diptera: Simuliidae) of the agricultural areas of Manitoba, Saskatchewan, and Alberta. Proc. X. Intern. Congr. Entomol. 3: 819–23.

**Lowther, J. K., and D. M. Wood.** 1964. Specificity of a black-fly, *Simulium euryadminiculum* Davies, towards its host, the common loon. Can. Entomol. 96: 911–13.

**Malloch, J. R.** 1914. American black flies or buffalo gnats. USDA, Bur. Entomol. Tech. Ser. 26: 1–82.

**Moore, H. S., IV, and R. Noblet.** 1974. Flight range of *Simulium slossonae,* the primary vector of *Leucocytozoon smithi* of turkeys in South Carolina. Environ. Entomol. 3: 365–9.

**Richmond, R. C.** 1972. Enzyme variability in the *Drosophila willistoni* group. III. Amounts of variability in the superspecies *D. paulistorum.* Genetics 70: 87–112.

**Ridgway, G. J., S. U. Sherburne, and R. D. Lewis.** 1970. Polymorphism in the esterases of Atlantic herring. Trans. Amer. Fish. Soc. 99: 147–51.

**Rogers, J. S.** 1972. Measures of genetic similarity and genetic distance. Univ. Texas Publ. 7213: 145–53.

**Rubtsov, I. A.** 1964. Mode and range of black fly (Diptera: Simuliidae) larval migrations. Entomol. Rev. 43: 27–33.

**Shaw, C. R., and R. Prasad.** 1970. Starch gel electropheresis of enzymes—a compilation of recipes. Biochem. Genet. 4: 297–320.

**Snoddy, E. L., and L. S. Bauer.** 1977. *Simulium* (Phosterodos) *penobscotensis,* a new species of black fly (Diptera: Simuliidae) from Maine, U.S.A. J. Med. Entomol. (In press).

**Stone, A., and E. L. Snoddy.** 1969. The black flies of Alabama (Diptera: Simuliidae). N.Y. State Mus. Bull. 349: 1–44.

**Townson, H.** 1976. Enzyme polymorphisms in vectors of disease—its study and interpretation of results: Studies of enzymes in the *Simulium damnosum* complex and *Aedes scutellaris* group. WHO publ. VBC/SC/76,21.