

DISCRIMINATION BETWEEN TWO TYPES OF FRESHWATER SPONGES USING
ALLOZYME ELECTROPHORESIS

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the
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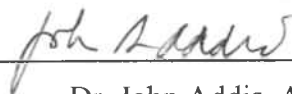
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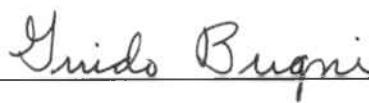


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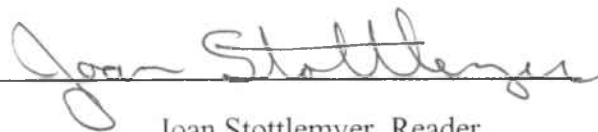
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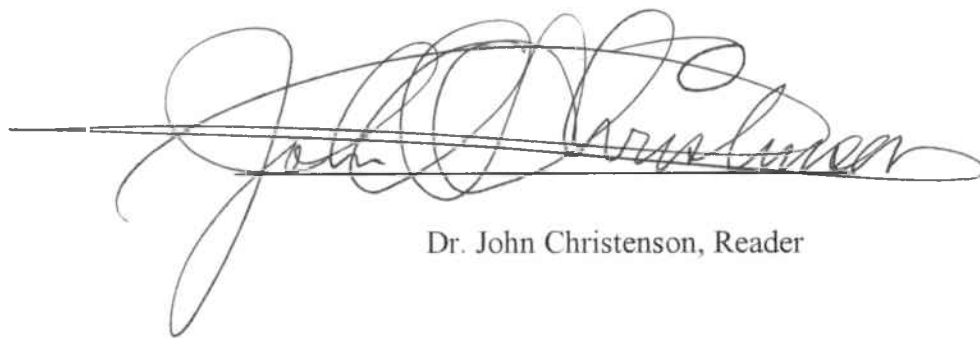
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ABSTRACT

Allozyme electrophoresis was used to discriminate between two types of freshwater sponges that previously could not be distinguished using morphological criteria. One of these types was a well-characterized species, *Ephydatia muelleri*, whereas the other could not be identified because of an absence of gemmules and gemmoscleres, structures on which classification of freshwater sponges largely depends. Homogenates prepared from five individuals of each sponge type were electrophoresed in cellulose acetate gels. The gels were then stained to localize bands of enzyme activity. Analyzing the stained gels revealed migration rates of hexokinase and sorbitol dehydrogenase that differed between the two sponge types. The absence of any electrophoretic bands common to the two sponge types argues that the sponges are different species.

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INTRODUCTION AND LITERATURE REVIEW

Several types of freshwater sponges have been identified in western Montana. A recent study (Barton and Addis, 1997) revealed two types of sponges that share the same habitat. One was identified as *Ephydatia muelleri*, but the other could not be identified due to the absence of gemmules and gemmoscleres in more than 60 collected specimens (Addis, unpublished observation). Gemmules (asexual propagules) and gemmoscleres (spicules associated with the gemmules) are key characteristics used for the classification of freshwater sponges. These two sponges exhibit some morphological differences in spicule morphology, in color, and in growth pattern, but their lack of microscleres combined with the lack of gemmules in the unidentified sponge ruled out the conclusion that these sponge types belong to separate species.

The Problem

The following information has been adapted from Barton and Addis (1997) unless otherwise noted. Morphological comparison revealed similarities between *E. muelleri* and the unidentified sponge (more similarities than with any other sponge identified in the study). For instance, the megascleres of the unidentified sponge overall were similar to those of *E. muelleri*. Both have megascleres that are slightly curved, sharply pointed at both ends, and covered with short spines except at their tips, and these similarities suggested that the unidentified sponge could be a variant of *E. muelleri*.

However, the presence of morphological differences in color, growth pattern, and spicule morphology between these two sponge types suggested otherwise. *E. muelleri* is usually green in color whereas the unidentified sponge is usually light tan to white in color. And although found in the same area, these sponges show sharp growth

boundaries upon contacting one another, indicating that their cells are not compatible (Addis, personal communication). In addition, despite similarity in overall morphology, the megascleres are significantly wider in the unidentified sponge than in *E. muelleri*, and scanning electron micrographs show that the spines of the unidentified sponge are uniformly low and grouped as rosettes, a characteristic which is not seen in *E. muelleri* (Addis, unpublished observation).

Is the unidentified sponge the same species as *E. muelleri* or does it belong to a distinct species? In order to answer this question, it was proposed to use allozyme electrophoresis to survey for genetic differences that could provide the genetic evidence needed to conclude that more than one species is present in the sampled population of sponges.

The Use of Allozyme Electrophoresis

Electrophoresis was developed as an analytical tool for biological and chemical research, and was chosen as the tool for this study of sponges. The following information has been modified from Richardson et al. (1986) unless otherwise indicated. A specific type of electrophoresis, isozyme electrophoresis, is designed to show the presence of a particular enzyme at more than one migration position. Allozymes are a specific type of isozyme that occur due to allelic variation at an enzyme locus, and allozyme electrophoresis has a variety of applications such as genetic identification, population analysis, species boundary determination, and phylogenetic reconstruction. This study utilized allozyme electrophoresis in a species boundary determination study to discern *E. muelleri* and the unidentified sponge as separate species.

According to Richardson et al. (1986), in a situation of cryptic species, allozyme electrophoresis is a very powerful tool, and it is rare that different species are reported as electrophoretically indistinguishable. Using allozyme electrophoresis allows the detection of fixed genetic differences, and a single difference is sufficient to recognize two co-existing cryptic species, as it shows that interbreeding is not occurring. This method has been used in other similar cryptic species studies. For instance, isozyme electrophoresis was used to differentiate cryptic species of spotted frogs (Green et al., 1997). This study used isozyme electrophoresis to discern two species of spotted frogs that could not be positively distinguished on the basis of standard defining morphological characteristics. Although the two types of frogs are virtually indistinguishable upon comparison of characteristics such as lengths of fingers, toes, and leg bones, discrepancies at four head measurements (orbital diameter, interorbital distance, tympanic diameter, internarial distance) suggested the presence of more than one species. Green et al. (1997) confirmed this suggestion using allozyme electrophoresis to elucidate fixed differences between specific allozymes that were chosen for analysis. Consequently, *Rana pretiosa*, originally recognized as one species, was found to be a complex of at least two species. Another example of the use of allozyme electrophoresis is the study of kangaroos concluded by Baverstock et al. (1984). This research elucidated electrophoretic differences between two populations (one from coastal New South Wales and one from the West Coast of Australia) of kangaroos that were greater than expected for allopatric populations of the same species. Consequently, *Sminthopsis murina*, originally recognized as a single species became recognized as five different species.

There are several methods for executing allozyme electrophoresis including starch gel electrophoresis, acrylamide gel electrophoresis, agarose gel electrophoresis, and cellulose acetate gel electrophoresis. Although starch gels are used more widely, there are certain advantages of using cellulose acetate gels as described by Richardson et al. (1986). These advantages include the following: (1) cellulose acetate gels require much less stain than starch gels (only 2-3 mL vs. 20-100 mL), thereby reducing the cost of the experimental run; (2) a smaller amount of tissue homogenate is required for electrophoresis; (3) the cellulose gels are premade, simplifying the electrophoretic procedure; (4) the time required for an electrophoretic run is less than that for starch gels (0.5-2 hr vs. overnight); (5) less time is required for staining; (6) lower voltages are required, lessening the need for an elaborate power supply and lessening the danger of electrical shock; and (7) cellulose gels are easy to handle, being durable and flexible.

Hypothesis Testing

The tested hypothesis was that the sample of sponges (five *Ephydatia muelleri*, five unidentified) consists of more than one species. Consequently, the null hypothesis tested was that the two apparent sponge types belong to a single interbreeding species, which is in Hardy-Weinberg equilibrium.

The Hardy-Weinberg equilibrium distribution predicts the expected genotypic proportions of homozygotes and heterozygotes based on the frequencies of two different alleles (p and q) of a particular gene locus, such that $p + q = 1$ and $p^2 + 2pq + q^2 = 1$. The expected proportions of homozygotes of an allele, $A_1 = p^2$, of an allele, $A_2 = q^2$ and the expected number of heterozygotes = $2pq$. Furthermore, in a sample of a single interbreeding species, with two alleles A_1 and A_2 , we would expect the following

genotypic proportions: $A_1A_1 = p^2$, $A_2A_2 = q^2$, and $A_1A_2 = 2pq$. Observed deviations from these expected proportions, such as the absence of heterozygotes, warrant suspicion of the nature of the species under study.

The probability of obtaining no heterozygotes in a supposed sample of five A_1A_1 individuals and five A_2A_2 individuals of a single interbreeding species is given by $(p^2 + q^2)^n$, where n = sample size. We can estimate the allele frequencies here as $p = 0.5$ and $q = 0.5$ since ten alleles in 20 total alleles of a particular gene gives 0.5. Thus, the probability of not obtaining any heterozygotes in a sample size of ten is only 0.1%. Consequently, the absence of heterozygotes in a supposed single species points to a non-interbreeding population and indicates that at least two species are present.

MATERIALS AND METHODS

Collection of Samples and Preparation of Tissue Homogenates

Five samples of each sponge type were collected from two western Montana lakes, Salmon Lake and Coopers Lake, on August 10, 1997. Although *E. muelleri* and the unidentified sponge were present in both lakes, *E. muelleri* was more abundant in Salmon Lake and the unidentified sponge was more abundant in Coopers Lake. To avoid depleting either lake of the less common sponge, samples were taken from the lake in which the particular type was more abundant. Although the sample size was only ten, this was the size suggested by Richardson et al. (1986). Several allozymes can be compared using a small number of individuals, and the presence of at least one fixed difference is strong evidence for distinct species.

The experimental protocol is summarized in Fig. 1, and the following procedure was adapted from Richardson et al. (1986) unless otherwise noted. After being kept in filtered lake water for 24 hrs to deplete the sponges of ingested food particles, the sponges were cut into smaller samples and weighed. Thirty-percent (w/v) homogenates were prepared in a homogenizing medium consisting of 0.1% β -mercaptoethanol and 0.02% Triton X-100 detergent. Samples were manually homogenized in ice-cold fritted glass homogenizers, using 20 up-down strokes of the pestle. The samples were centrifuged in 15-mL glass centrifuge tubes for 30 min at 10,780 X g. The supernatant was removed and stored in one mL aliquots in microtubes in a -70° C freezer.

COLLECT AND MAINTAIN SPONGES IN FILTERED LAKE WATER FOR 24 HRS



HOMOGENIZE TISSUE IN 0.1 % β -MERCAPTOETHANOL AND 0.02% TRITON-X-100 DETERGENT



ELECTROPHORESE IN CELLULOSE ACETATE STRIPS



STAIN GEL STRIPS



SCORE GELS



ANALYZE BANDING PATTERNS

Fig. 1. Flowchart of experimental procedures.

Electrophoresis

A total of 11 enzymes were surveyed, and for each, the buffer recommended by Richardson et al. (1986) was used (Table 1). The buffers used were as follows: 0.02 M phosphate, pH 7.0, and 0.05 M Tris-maleate, pH 7.8. Cellulose acetate strips (Gelman) were soaked in buffer for approximately 20 min, prior to use. After soaking, the gels were removed from the soaking tank using either forceps or latex-gloved hands and transferred to the electrophoretic chamber. The chamber contained approximately 700 mL of the respective buffer. The ends of the strips were submerged in the buffer and secured with magnets. Next, using a micropipette the gels were loaded with 3 μ l of the homogenate from *Ephydatia muelleri* or the unidentified sponge. In some cases, a 1:1 mix of the two homogenates was loaded as well. After loading, the lid was placed on the electrophoretic chamber, and the gel was electrophoresed at 225 volts for 25 min (in a few cases, electrophoresis was carried out for longer time periods).

Table 1. Enzymes and buffers used in analysis. A, 0.05 M Tris-maleate, pH 7.8; B, 0.02 M phosphate, pH 7.0

ENZYME	BUFFER
Acid Phosphatase (ACP)	B
Alcohol Dehydrogenase (ADH)	B
Alkaline Phosphatase (AK)	B
Glucose Dehydrogenase	B
Glutamate Dehydrogenase	A
Glucose Phosphate Isomerase (GPI)	A or B*
Hexokinase (HK)	B
Lactate Dehydrogenase (LDH)	A
Malate Dehydrogenase (MDH)	B
Sorbitol/ L-iditol dehydrogenase (SORDH)	A or B*
Superoxide dismutase (SOD)	A

*Buffer B was more effective in producing sufficient resolution for analysis

Staining

At the end of each electrophoretic run, the gels were immediately stained, exploiting the activities of the enzymes present. The enzymes surveyed and the composition of the staining media are as follows:

Acid phosphatase: 1.25 mL 0.05 M citric acid (pH 4.0), 2.5 mg α -naphthyl acid phosphate, 3 mg Fast Garnet GBC.

Alcohol dehydrogenase: 1.0 mL 0.1 M Tris-HCl, pH 8.0, 50 μ L NAD stock (25 mg/mL), 50 μ L stock phenazine methosulfate (PMS) (2 mg/mL), 50 μ L stock methyl-thiazolyl blue (MTT) (6 mg/mL), 50-100 μ L ethanol.

Alkaline phosphatase: 1.0 mL Tris-HCl (pH 8.6), 50 μ L 1.0 M $MgCl_2$, 5 mg β -Naphthyl acid phosphate, 4 mg Fast Blue BB.

Glucose dehydrogenase: 250 mg glucose, 1.5 mL 0.1 M Tris-HCl (pH 8.6), 50 μ L NAD stock (25 mg/mL), 50 μ L MTT stock (6mg/mL), 50 μ L stock PMS (2mg/mL).

Glutamate dehydrogenase: 10 mg D-L glutamic acid, 1 mL 0.1 M Tris-HCl (pH 8.0), 50 μ L NAD stock (25mg/mL), 50 μ L MTT stock (6 mg/mL), 50 μ L PMS stock (2mg/mL).

Glucose phosphate isomerase: 1.0 mL Tris-HCl (pH 8.0), 50 μ L NAD stock (25 mg/mL), 50 μ L 1.0 M $MgCl_2$, 50 μ L (PMS) stock (2 mg/mL), 50 μ L (MTT) stock (6

mg/mL), 2.5 mg fructose-6-phosphate, 2.5 μ L glucose-6-phosphate dehydrogenase stock (400 U/mL), added just before use.

Hexokinase: 1.0 mL 0.1 M Tris-HCl, pH 8.6, 50 μ L 1.0 M $MgCl_2$, 3 mg ATP, 1 mg glucose, 50 μ L NAD stock (25 mg/mL), 50 μ L PMS stock (2 mg/mL), 50 μ L MTT stock (6 mg/mL), 2.5 μ L glucose-6-phosphate dehydrogenase stock (400 U/mL), added just before use.

Lactate dehydrogenase: 1.0 mL Tris-HCl (pH 8.0), 50 μ L NAD stock (25 mg/mL), 50 μ L PMS stock (6mg/mL), 50 μ L MTT stock (2 mg/mL), 50 μ L lactate stock (50mg/mL L-lactic acid, pH 8.0).

Malate dehydrogenase: 1.0 mL 0.1 M Tris-HCl, pH 8.0, 50 μ L NAD stock (25 mg/mL), 50 μ L PMS stock (2 mg/mL), 50 μ L MTT stock (6 mg/mL), 100 μ L malate stock (50 mg/mL L-malic acid, pH 8.0).

Sorbitol/L-iditol dehydrogenase: 1.0 mL 0.1 M Tris-HCl, pH 8.0, 50 μ L NAD stock (25 mg/mL), 50 μ L PMS stock (2 mg/mL), 50 μ L MTT stock (6 mg/mL), 7.5 mg Sorbitol.

Superoxide dismutase: 1.0 mL 0.1 M Tris-HCl (pH 8.0), 50 μ L PMS stock (2 mg/mL), 50 μ L MTT stock (6mg/mL).

Reagents were obtained from Sigma Chemical Company.

To stain the gels, an overlay method was used. Gel strips were soaked in the particular stain until saturated (about 30 sec), and this strip was laid onto the gel strip that was used for the electrophoretic run, ensuring the absence of air bubbles by carefully smoothing the two strips together with a glass plate or with latex-gloved hands. These sets of strips were sealed from the air using plastic wrap before they were placed in the dark for development. The wrapped strips were checked after approximately five min, and at five min intervals thereafter until bands appeared. The strips were removed from the wrap and fixed in 50% glycerol if the stain contained MTT (Hillis et al., 1996). Otherwise the strips were fixed in acetic acid.

Analysis

Each stained gel was examined for the presence of heterozygotes and to determine the mobility of the bands. Heterozygotes could be identified by the presence of two bands whereas homozygotes produced only a single band. To ensure that the stained bands were different from one another, two methods were used. First, the relative mobility of each band was measured. This number was the distance (in mm) from the cathodal origin to the middle of each band divided by the time of the electrophoretic run, yielding the rate of movement (mm/min). Second, the two types of homogenates were mixed 1:1 and electrophoresed. These gels were examined for distinct bands, each representative of one of the two sponge types.

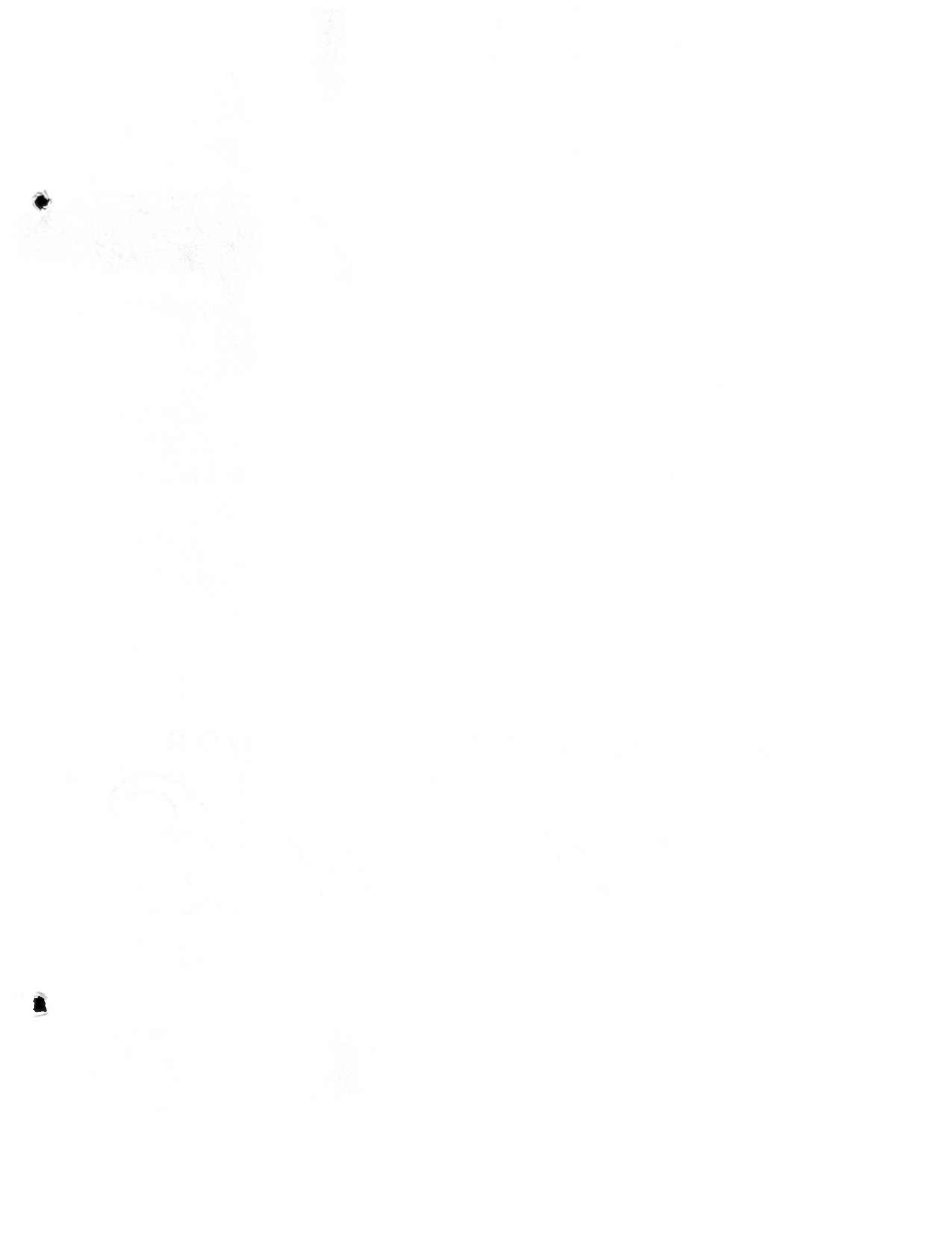
RESULTS AND DISCUSSION

Fig. 2 shows the two sponge types studied, and the results of gel analysis on these sponge types are summarized in Table 2. Of the eleven loci surveyed, five showed resolutions sufficient for analysis. Of these five loci, two diagnostic loci, hexokinase (HK) and sorbitol dehydrogenase (SORDH), showed differing band mobility between *E. muelleri* and the unidentified sponge. Electrophoresis of the 1:1 mixture of homogenate resulted in distinct bands for each of the sponges at the HK and SORDH loci. Different band migration rates were also observed for the alcohol dehydrogenase (ADH) and glucose phosphate isomerase (GPI) (Fig. 3), but these differences were not confirmed by electrophoresis of the 1:1 homogenate mixture.

The results illustrate that *E. muelleri* and the unidentified sponge have fixed differences, or no shared alleles, at two loci, HK and SORDH. Distinct bands from the 1:1 homogeneous mix confirmed these fixed differences at HK and SORDH.

Perhaps the most significant finding was the absence of double bands (excluding bands resulting from electrophoresis of the 1:1 mixture) on the HK and SORDH stained gels. This showed a lack of heterozygotes at these two allelic types among the ten sponges sampled. As explained above, the probability of not obtaining any heterozygotes in a sample of ten of an assumed single interbreeding species is only 0.1%. Furthermore, this probability is compounded since there are two instances of a lack of heterozygotes (HK and SORDH) such that $(0.1\%)^2 = 0.0001\%$. Since this probability is so low, the null hypothesis can be rejected with certainty, thereby supporting the tested hypothesis that *Ephydatia muelleri* and the unidentified sponge are distinct species.

This finding is complicated by the presence of symbiotic algae in *E. muelleri*. Despite the attempt to deplete the sponges of ingested food particles, *E. muelleri* retained the symbiotic algae. Consequently, it is possible that the enzymes analyzed were algal enzymes instead of *Ephydatia muelleri*. Attempts were made to test this possibility, but



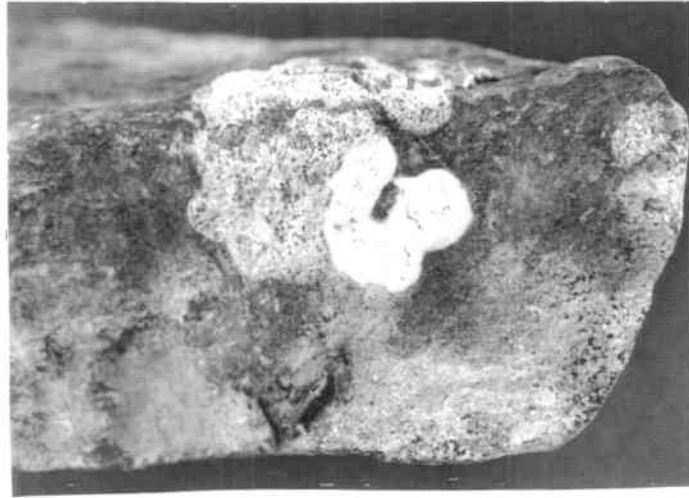
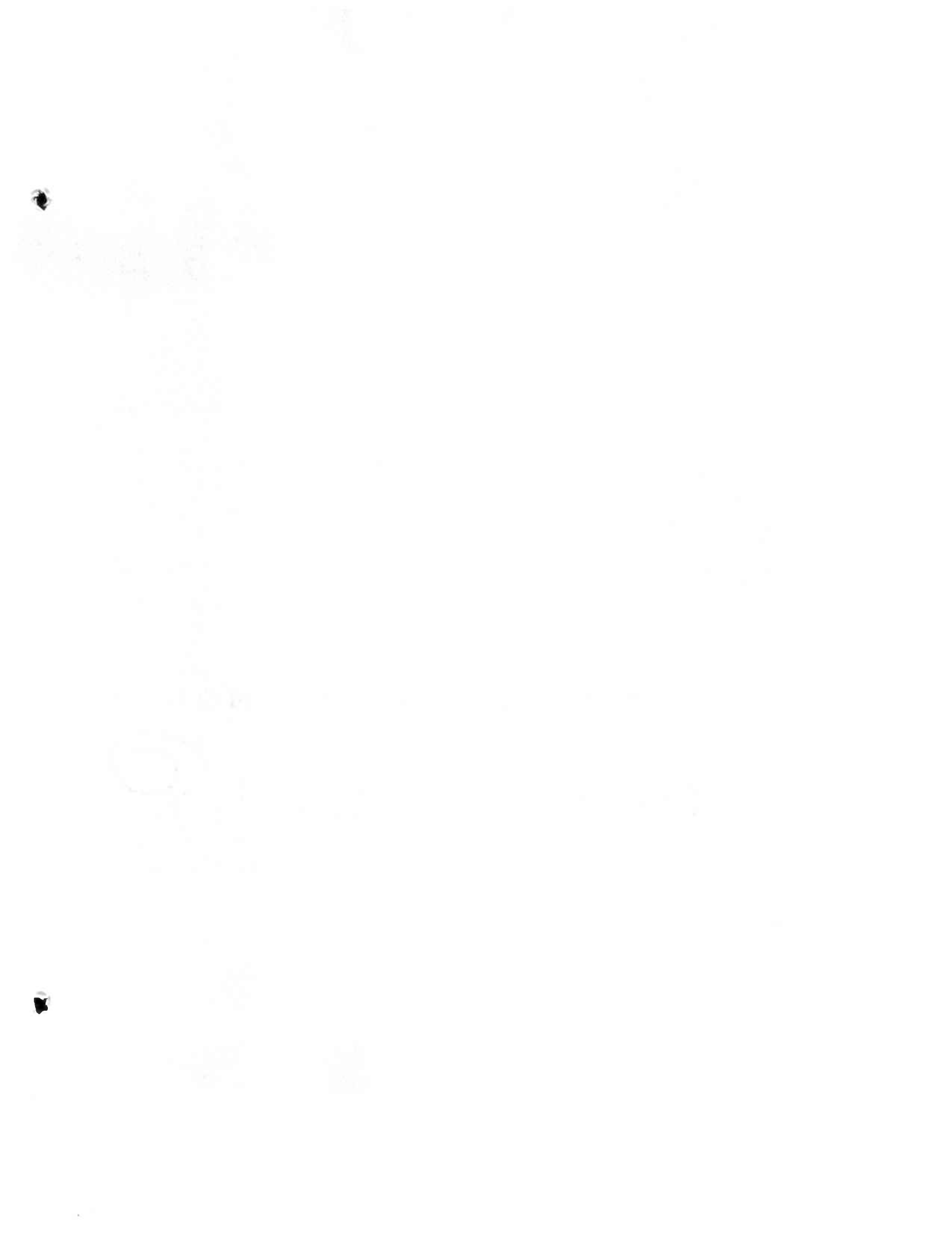


Fig. 2. *Ephydatia muelleri* and the unidentified sponge encrusting a rock surface. *E. muelleri* (tan) and the unidentified type (white) form a sharp boundary where they contact one another, suggesting that minimally they represent distinct strains of a single species. Dried specimen from Salmon Lake.

Table 2. Results of allozyme electrophoresis. Bold type indicates diagnostic alleles. +, differing band mobility for the two sponge types; -, equivalent band mobility in the two sponges; 0, resolution too low for analysis; *, although differing banding mobility was observed, it was not confirmed through electrophoresis of a 1:1 mixture of homogenates.

ENZYME	RESULT
Acid phosphatase	0
Alcohol dehydrogenase	*
Alkaline phosphatase	0
Glucose dehydrogenase	0
Glutamate dehydrogenase	0
Glucose phosphate isomerase	*
Hexokinase	+
Lactate dehydrogenase	0
Malate dehydrogenase	-
L-idoitol/(sorbitol) dehydrogenase	+
Superoxide dismutase	0



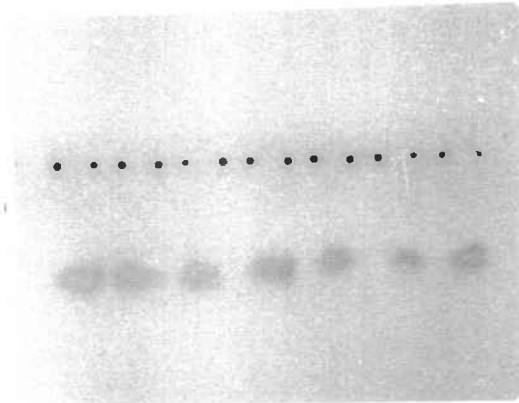


Fig 3. Gel showing stained GPI bands. Dotted line indicates origin; lanes 1-3, bands representative of the unidentified sponge homogenate; lane 4, single band resulting from electrophoresis of 1:1 mixture of homogenates (single band could actually be two bands too close together to resolve a difference between them); lanes 5-6, bands representative of *E. muelleri*. Bands from *E. muelleri* have different mobility than bands from the unidentified sponge type.

the results were inconclusive. However, the possibility that the algal enzymes an important factor seems unlikely since one would expect two bands if both algal and sponge enzymes were present in large quantity whereas only single bands were observed on the stained gels.

In addition, more research needs to be done to determine whether additional enzymes like ADH and GPI (Table 2) also exhibit fundamental genetic difference between the two sponge types. According to Richardson et al. (1986), at least two fixed differences or supporting morphological correlates are needed to confirm the presence of two species.

Barring the possibility that the results are due to the abundance of algal enzymes which matched sponge enzymes in their electrophoretic mobilities, it can be concluded that the unidentified sponge is a species distinct from *Ephydatia muelleri*.

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