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EXPERIMENTAL TAXONOMY OF *BULINUS* (GASTROPODA: PLANORBIDAE)

II. RECIPES FOR HORIZONTAL STARCH GEL ELECTROPHORESIS OF TEN ENZYMES IN *BULINUS* AND DESCRIPTION OF INTERNAL STANDARD SYSTEMS AND OF TWO NEW SPECIES OF THE *BULINUS FORSKALII* COMPLEX

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SUMMARY

The problems encountered in the identification of African freshwater snails of medical importance have resulted in a search for new and more standardized characteristics. The methods described for the horizontal starch gel electrophoresis of single individuals of *Bulinus* make it possible to score the phenotype of ten different enzymes in the individual. A system of standards using the enzyme phenotype of *Bulinus truncatus* as reference is described and brief mention is made of the promising results obtained so far by the application of the method on the taxonomy and identification of *Bulinus* species from Kenya.

INTRODUCTION

The identification of freshwater snails of medical importance is difficult because of the variation encountered in the anatomical and morphological characteristics commonly used for separating species. This great variability can be found within small geographical areas and is due to the influence of ecological conditions on the characteristics. New characteristics, which could be useful for the separation of *Biomphalaria* and *Bulinus* species, such as cytological and biochemical ones, have been searched for. The biochemical and cytological areas of taxonomy together with infection experiments and other possible characteristics that require live material for the investigations are called experimental taxonomy. However, experimental taxonomy is not an alternative to but merely an extension of classical taxonomy, and makes use of the new developments in biological sciences.

The cytological characteristics, *i.e.*, the chromosome number, have yielded little information apart from a useful contribution to the taxonomy of some species of *Bulinus*. In *Bulinus*, four different chromosome numbers are found[1]. The most

common chromosome number ($2n = 36$) is found in all investigated members of the *forskalii* and the *africanus* groups and in most members of the *tropicus–natalensis–truncatus* group. The tetraploid chromosome number ($2n = 72$) is found in most populations of *Bulinus truncatus*, in *Bulinus guernei*, in *Bulinus permembranaceus* and possibly in *Bulinus coulboisi*. Hexaploid ($2n = 108$) and octoploid ($2n = 144$) chromosome numbers have so far been found only in some Ethiopian populations of the *tropicus–natalensis–truncatus* group.

The biochemical characteristics obtainable by protein or enzyme electrophoresis have contributed little to the taxonomy of *Bulinus* and *Biomphalaria*. Studies have been carried out using esterase in *Bulinus*[2–6] and in *Biomphalaria*[7–9] on laboratory material. Isoenzyme data from natural populations have been published for *Biomphalaria*[10–13] and for *Bulinus*[14–16]. The electrophoresis of egg proteins has been useful in distinguishing between *Bulinus truncatus* and the other members of the *tropicus–natalensis–truncatus* group[17,18].

One of the main reasons for the limited success of electrophoresis in the taxonomy of freshwater snails compared with the success obtained in other animal and plant groups is that the studies have not been carried out at the population level and mostly only one electrophoretic characteristic has been investigated. The necessity to use several electrophoretic characteristics at the population level in the improvement of *Bulinus* and *Biomphalaria* taxonomy has led to the development of the standard electrophoretic procedures described in this paper, through which any single Bulinid snail can be surveyed for a total of ten enzymes.

EXPERIMENTAL

Preparation of samples for electrophoresis

After the water adhering to the shell has been blotted off, the specimen is placed in a micro-test-tube where homogenization takes place without addition of water. This results in a concentrated sample. A dilute sample is obtained by cleaning the piston of the homogenizer in another micro-test-tube to which 10–25 μl of water have been added. After centrifugation at 18,000 g for 2 min the samples are ready for application to the gel by sucking up the supernatant with 6 × 3 mm pieces of Whatman No. 1 chromatography paper.

Electrophoretic conditions

Electrophoresis was performed at 2° in Plexiglass frames with inside dimensions of 15 × 24 × 0.6 cm using 10% Connaught hydrolysed starch in the appropriate gel buffer. The solidified gels were allowed to cool in a refrigerator for 1.5 h. It has been observed that too long a cooling period has an adverse effect on the electrophoresis. The compositions of the gel and bridge buffers are given in Table I. The samples were applied in a slit cut 2–3 cm from the edge of the gel.

In buffer A electrophoresis was performed with dilute samples for esterase and phosphoglucose isomerase staining. With concentrated samples, 3-hydroxybutyrate dehydrogenase can be assayed. The duration of the electrophoresis was 3.5 h at 320 V and 50 mA.

In buffer B electrophoresis was performed with concentrated samples for 3.5 h at 280 V and 45 mA and the enzymes that would be stained were glutamate-

TABLE I

BUFFERS USED FOR ELECTROPHORESIS OF *BULINUS*

Amounts are given in grams per litre.

Buffer system	*Boric acid*	*Tris*	*Titriplex II*	*Citric acid·H_2O*	*Sodium hydroxide*	*pH*
A: Bridge	4.64	12.72	0.06	—	—	8.6
Gel	0.680	1.817	0.585	—	—	8.1
B: Bridge	30.95	—	—	—	*ca.* 1.4	Adjust to 6.9
Gel	—	9.2	—	0.96	—	8.7
C: Bridge	4.86	31.62	1.23	—	—	9.0
Gel	0.54	10.54	0.41	—	—	9.0

oxaloacetate transaminase, nucleoside phosphorylase, xanthine dehydrogenase and NADP-dependent isocitrate dehydrogenase.

In buffer C electrophoresis was performed with concentrated samples for 3.5 h at 280 V and 50 mA. Staining was carried out for the enzymes fructokinase, α-glycerophosphate dehydrogenase, 3-hydroxybutyrate dehydrogenase and 6-phosphogluconate dehydrogenase.

After completion of the electrophoresis, the application papers were removed and the gel was cut horizontally into two halves. Detection of enzyme activity took place on the cut surface.

Staining procedures

All enzyme staining took place in the dark at 37°.

Esterase. A 250-ml volume of 0.009 *M* phosphate buffer (pH = 7.3), 100 mg of Fast Red TR and 2 ml of 1% α-naphthyl acetate in acetone.

Glutamate–oxaloacetate transaminase. A 3.8-g amount of dipotassium hydrogen orthophosphate (K_2HPO_4), 1 g of polyvinylpyrrolidone, 100 mg of Titriplex II, 74 mg of 2-oxoglutaric acid and 266 mg of L-aspartic acid in 100 ml water. Just before use, 250 mg of Fast Violet B salt was added.

From stock solutions each containing 5 mg/ml of chemical, the amounts given below of NAD, NAPD, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide] and PMS (phenazine methosulphate) were added. The following staining mixtures all contained 2.5 mg of MTT and 1.25 mg of PMS and, just before pouring the stain on to the gel, 25 ml of 2% agarose in 0.025 *M* magnesium chloride solution at 60° were added.

Phosphoglucose isomerase. A 10-mg amount of fructose-6-phosphate, 7 U (units) of glucose-6-phosphate dehydrogenase and 2.5 mg of NADP in 25 ml of 0.06 *M* Tris–hydrochloric acid (pH 8.0).

3-Hydroxybutyrate dehydrogenase. A 100-mg amount of 3-hydroxybutyrate and 2.5 mg of NAD in 25 ml of 0.06 *M* Tris–hydrochloric acid [pH 8.0 (buffer A) or pH 6.9 (buffer B)].

Fructokinase. A 100-mg amount of fructose, 50 mg of adenosine-5′-triphosphate, 70 U of phosphoglucose isomerase, 7 U of glucose-6-phosphate dehydrogenase and 2.5 mg of NADP in 25 ml of 0.06 *M* Tris–hydrochloric acid (pH 6.9).

α-Glycerophosphate dehydrogenase. A 100-mg amount of α-glycerophosphate and 2.5 mg of NAD in 25 ml of 0.06 *M* Tris–hydrochloric acid (pH 6.9).

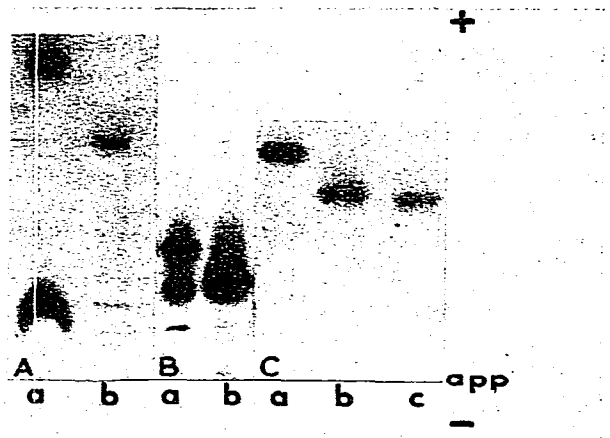

Fig. 1.

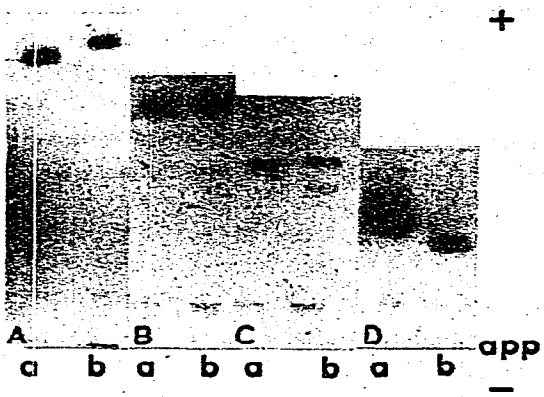

Fig. 2.

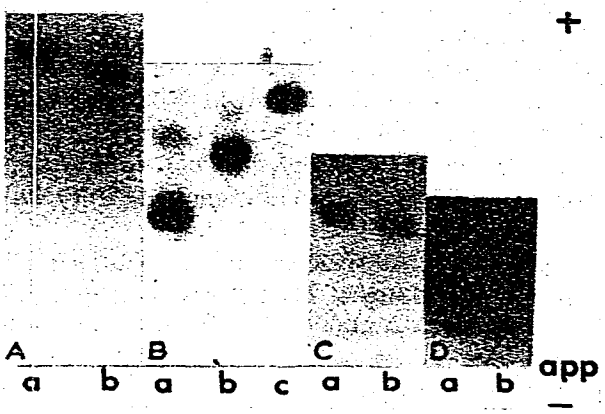

Fig. 3.

6-Phosphogluconate dehydrogenase. A 20-mg amount of 6-phosphogluconate and 2.5 mg of NADP in 25 ml of 0.06 *M* Tris–hydrochloric acid (pH 6.9).

NADP-dependent isocitrate dehydrogenase. A 100-mg amount of isocitrate and 2.5 mg of NADP in 25 ml of 0.06 *M* Tris–hydrochloric acid (pH 6.9).

Xanthine dehydrogenase. A 30-mg amount of hypoxanthine and 2.5 mg of NAD in 25 ml of 0.06 *M* Tris–hydrochloric acid (pH 6.9).

Nucleoside phosphorylase. A 50-mg amount of inosine and 0.4 U of xanthine oxidase in 25 ml of 0.009 *M* phosphate (pH 7.3).

Procedure of internal standards

In order to be able to compare the electrophoretic mobilities of different populations run at different times, it is desirable to use a system of standards with known electrophoretic mobility. In a previous study[15], it has been shown that both the esterases and the phosphoglucose isomerases of *Bulinus truncatus* display a remarkably low geographical variation, and the same observation has been made on 3-hydroxybutyrate dehydrogenase (personal observation). This very low geographical variation could probably be observed in the other enzymes not yet properly investigated in this species. This makes the isoenzymes of *Bulinus truncatus* very suitable as reference enzymes in a comparative investigation of *Bulinus*. Further, *Bulinus truncatus* is found in most of Africa except the southernmost part, *i.e.*, south of Angola and Malawi.

The electrophoretic mobilities in enzymes of the stock designated *Bulinus truncatus*. Imbaba, Egypt, collected 11-9-1971 and maintained at the Danish Bilharziasis Laboratory, have arbitrarily been assigned a relative mobility (*rm*) of 1.00. All other electrophoretic mobilities are and will be given as rm-values to these enzymes. A designation of esterase standard is in press[15]. For the enzyme 3-hydroxybutyrate dehydrogenase a triple banded structure is normally observed in *Bulinus truncatus*. The reference band with relative mobility 1.00 in this instance is the least anodic of the three bands which appoints the fast band with *rm* 1.22 in buffer A and 1.18 in buffer B.

Fig. 1. Starch gel isoenzyme pattern of whole animal extracts using buffer A. (A) 3-Hydroxybutyrate dehydrogenase of (a) *Bulinus globosus*, Tanga area, Tanzania, and (b) *Bulinus tropicus*, Thika area, Kenya. (B) Esterases of (a) *Bulinus guernei*, Thies, Senegal, and (b) *Bulinus truncatus*, Senegal. (C) Phosphoglucose isomerase of (a) *Bulinus globosus*, Coast Province, Kenya, (b) *Bulinus nasutus*, Coast Province, Kenya, and (c) *Bulinus truncatus*, Imbaba, Egypt.

Fig. 2. Starch gel isoenzyme pattern of whole animal extracts using buffer B. (A) NADP–isocitrate dehydrogenase of (a) *Bulinus tropicus*, Eldoret, Kenya, and (b) *Bulinus permembranaceus*, Njabini, Kenya. (B) Nucleoside phosphorylase of (a) *Bulinus ugandae*, Dunga, Kenya, and (b) *Bulinus africanus*, Misungwi, Tanzania. (C) Xanthine dehydrogenase of (a) *Bulinus africanus*, Misungwi, Tanzania, and (b) *Bulinus ugandae*, Dunga, Kenya. (D) Glutamate–oxaloacetate transaminase of (a) *Bulinus tropicus*, Molo, Kenya, and (b) *Bulinus permembranaceus*, Njabini, Kenya.

Fig. 3. Starch gel isoenzyme pattern of whole animal extracts using buffer C. (A) Fructokinase of (a) *Bulinus nasutus*, Coast Province, Kenya, and (b) *Bulinus tropicus*, Lake Sergoit, Kenya. (B) α-Glycerophosphate dehydrogenase of (a) *Bulinus forskalii*, Kisumu, Kenya, (b) *Bulinus barthi*, Mariakani Dam, Kenya, and (c) *Bulinus browni*, Obtuso, Kano Plain, Kenya. (C) 6-Phosphogluconate dehydrogenase of (a) *Bulinus truncatus*, Awassa, Ethiopia, and (b) *Bulinus octoploides*, Debra Sina, Ethiopia. (D) 3-Hydroxybutyrate dehydrogenase of (a) *Bulinus forskalii*, near Obtuso, Kenya, and (b) *Bulinus browni*, Bondo, Kenya.

RESULTS

The methods described above are the most reproducible electrophoretic methods obtained by staining for 32 enzymes in up to 13 buffer combinations. Some of the results obtained have already been published[14–16]. Figs. 1–3 show photographs of the zymograms. The photographs depict wherever possible differences between closely related species. In the enzyme α-glycerophosphate dehydrogenase it has been found that the species *Bulinus forskalii* actually is composed of three species, two of which are described below.

Bulinus browni n. sp.

Diagnosis. In morphology and anatomy it is very similar to *Bulinus forskalii* (Ehrenberg 1831), but a diagnostic characteristic is found in α-glycerophosphate dehydrogenase, where *Bulinus forskalii* has $rm = 0.91$ and *Bulinus browni* has $rm = 1.48$ (Fig. 3B, a and c).

Remarks. On the Kano Plain, Kenya, populations of *Bulinus forskalii* and *Bulinus browni* are found in between each other and no populations have shown the intermediate phenotype, *i.e.*, α-glycerophosphate dehydrogenase, $rm = 0.91/1.48$.

Distribution. Bulinus browni is known from Kano Plain, Kenya, and Salisbury, Rhodesia.

Holotype. Kenya, Nyanza Province, Obtuso, UTM ref. 36MYQ0085.

Paratypes. Same data as for the holotype.

Bulinus barthi n. sp.

Diagnosis. In morphology and anatomy it is very similar to *Bulinus forskalii* (Ehrenberg 1831), but a diagnostic characteristic is found in α-glycerophosphate dehydrogenase, where *Bulinus forskalii* has $rm = 0.91$ and *Bulinus barthi* has $rm = 1.22$ (Fig. 3B, a and b).

Remarks. On the coast of Kenya and Tanzania, populations of *Bulinus forskalii* and *Bulinus barthi* are found in between each other and no populations have shown the intermediate phenotype, *i.e.*, α-glycerophosphate dehydrogenase, $rm = 0.91/1.22$.

Distribution. Bulinus barthi is known from the coastal area of Kenya and Tanzania.

Holotype. Kenya, Coast Province, Mariakani Dam, UTM ref. 37MEF5374.

Paratypes. Same data as for the holotype.

DISCUSSION

The holotypes of *Bulinus browni* and *Bulinus barthi* are in the collection of the Danish Bilharziasis Laboratory. Of the *Bulinus forskalii* complex, a number of populations have been investigated by electrophoresis and from the other enzymes tested additional differences between the three species are found. A more comprehensive account of these data is in preparation.

The results published represent only a small part of the data obtained by an electrophoretic survey of natural populations of *Bulinus* from Kenya. Several papers dealing with the experimental taxonomy of the Bulinid subgroups are in

preparation. The data generally support the classical taxonomy, but some adjustments are necessary. In a broader perspective, it is the intention to survey a considerable number of natural populations of *Biomphalaria* and *Bulinus* in order to make the taxonomy of the genera more stable and at the same time provide new characteristics for the species identification. Work along these lines is in progress and any live material of *Biomphalaria* and *Bulinus* arriving at the laboratory will be gratefully received and used for the study. The material will be used to fill gaps in the knowledge of schistosome intermediate host taxonomy.

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