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Comparative Studies on Soluble Protein Profiles and Isozyme Patterns in 3 Related Species of the Genus *Diphyllobothrium*

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Abstract

Three *Diphyllobothrium* species, i.e. *D. nihonkaiense* Yamane *et al.*, 1986, *D. latum* (Linné, 1758), and *Diphyllobothrium* sp. ind. by Yazaki *et al.*, 1986 were examined in comparing the soluble protein profiles and isozyme patterns by isoelectric focusing in polyacrylamide gels. The species specific patterns were observed in the soluble protein profiles and the zymograms of 2 enzymes (esterase 1 and esterase D, acid phosphatase). The enzyme polymorphism was observed in the zymograms of malic enzyme and β-N-acetyl-glucosaminidase in *D. nihonkaiense*, and each of them was different from each pattern in the other 2 species. The isozyme patterns of 3 enzymes (6-phosphogluconate dehydrogenase, hexokinase, superoxide dismutase) in *D. latum* were different from those in the other 2 species, in which the common isozyme patterns were demonstrated. On the other hand *D. latum* and *Diphyllobothrium* sp. ind. indicated the similar isozyme pattern of mannosephosphate isomerase, being different from that of *D. nihonkaiense*, whereas *D. latum* and *D. nihonkaiense* indicated the similar isozyme pattern of adenylate kinase, being different from that of *Diphyllobothrium* sp. ind. In the zymogram of phosphoglucomutase enzyme polymorphism was observed in each of 3 species. Isozyme pattern of glucosephosphate isomerase was common in 3 species. On the basis of these data, the genetic relation between *D. latum* and *D. nihonkaiense* supposed to be more distant from that between *D. nihonkaiense* and *Diphyllobothrium* sp. ind.

Key words: *Diphyllobothrium latum*, *Diphyllobothrium nihonkaiense*, isozyme, soluble protein profile, isoelectric focusing, cestode

Introduction

The tapeworm as the cause of Japanese diphyllobothriasis, of which plerocercoid is found from cherry salmon, *Oncorhynchus masou*, or pink salmon, *O. gorbuscha*, has so far been regarded as *Diphyllobothrium latum* (Linné, 1758) without any taxonomic consideration. Kamo (1978, 1981) proposed the urgent necessity of reconsidering the taxonomic status of diphyllobothriid cestodes in Japan on the basis of the most advanced criteria. A revised taxonomical study of the so-called broad tapeworm in Japan in comparing with original *Diphyllobothrium latum* in Finland revealed differences in morphological and biological characteristics between them. Thus *D. nihonkaiense* was described as a new species by Yamane *et al.*, 1986.

Voge (1969) stressed the importance of the biochemical approach to problems encountered in cestode taxonomy. Bylund and Djupsund (1977) demonstrated each specific protein profile in 4 *Diphyllobothrium* species of Nothern Europe as a useful aid of species identification. Recently electrophoretic separation of isozymes has been successfully applied to the identification of the different species or strains of many helminths including cestodes

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(Le Riche and Sewell, 1977, 1978a, 1978b; McManus and Smyth, 1979; Macpherson and McManus, 1982; Baldock *et al.*, 1985). However, there is no report of isozymes in the genus *Diphyllobothrium*.

In the present investigation, 3 *Diphyllobothrium* species, i.e. *D. nihonkaiense* Yamane *et al.*, 1986, *D. latum* (Linné, 1758), and *Diphyllobothrium* sp. ind. originated from *Hypomesus pretiosus japonicus*, Japanese surf-smelt and *Osmerus eperlanus mordax*, olive rainbow smelt, were examined in comparing the soluble protein profiles and isozyme patterns by isoelectric focusing in polyacrylamide gels and then the genetic relationships of these species were reviewed on the basis of isozyme patterns.

Materials and Methods

Collection of specimens:

Mature proglottids of *D. latum*, (A) and (B), were a part of spontaneously expelled strobila from a voluntarily infected man on November 1982 and on July 1986, respectively (Kamo *et al.*, 1986).

Mature proglottids of *D. nihonkaiense* (C) was a part of spontaneously expelled strobila from a human case. Mature proglottids (D) was also a part of spontaneously expelled strobila from a man voluntarily infected with the plerocercoids of *D. nihonkaiense* from cherry salmon. Mature strobila of *D. nihonkaiense* raised in a golden hamster infected with the plerocercoids from a cherry salmon was divided into 2 parts, i.e. immature proglottids with the scolex (E) and mature proglottids with a part of immature proglottids (F).

Mature strobilae of *Diphyllobothrium* sp. ind., (G) and (H), were obtained from each golden hamster experimentally infected with the plerocercoids from *H. pretiosus japonicus* and *O. eperlanus mordax*, respectively.

These 8 specimens of 3 *Diphyllobothrium* species examined were listed in Table 1. Each specimen was washed several times with physiological saline and stored at −80°C until used.

Preparation of extracts for electrophoresis:

Each specimen was homogenized separately in 3 volumes of Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol using a Polytron PT 10 tissue homogenizer. The homogenates were centrifuged at 29,500 $\times g$ for 20 min and the supernatants were re-centrifuged at 100,000 $\times g$ for 1 hr. The final supernatants were used as the samples to detect the soluble protein profiles and isozymes by isoelectric focusing.

Table 1 The list of 8 specimens of 3 *Diphyllobothrium* species examined.

	Species	Part of strobila	Origin of plerocercoid	Host	Stored period
(A)	*D. latum* (from Finland)	mature proglottids	pike	human	3 years and 10 months
(B)	*D. latum* (from Finland)	mature proglottids	pike	human	4 months
(C)	*D. nihonkaiense*	mature proglottids	unknown	human	2 years and 10 months
(D)	*D. nihonkaiense*	mature proglottids	cherry salmon	human	11 months
(E)	*D. nihonkaiense*	immature proglottids with scolex	cherry salmon	golden hamster	11 months
(F)	*D. nihonkaiense*	mature proglottids with immatures	cherry salmon	golden hamster	11 months
(G)	*Diphyllobothrium* sp. ind.	strobila	Japanese surfsmelt	golden hamster	1 year and 6 months
(H)	*Diphyllobothrium* sp. ind.	strobila	olive rainbow smelt	golden hamster	1 year and 7 months

Electrophoresis:

Isoelectric focusing was performed in polyacrylamide gels using the LKB 2217 Ultrophore and 2297 Macrodrive 5 power supply at 10°C for 2 hr according to the method described by Bylund and Djupsund (1977). The limits for voltage, current and power were set at 1,200 V, 50 mA, 25 W. The 3 working modes (constant current, constant power, constant voltage) were changed by automatic crossover.

The plate was submerged immediately in the hot (60°C) fixative and proteins were stained with Coomassie Brilliant Blue R 250 by the method of Bylund and Djupsund (1977).

The enzymes examined were malic enzyme (ME, EC 1.1.1.40), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44), superoxide dismutase (SOD, EC 1.15.1.1), hexokinase (HK, EC 2.7.1.1), adenylate kinase (AK, EC 2.7.4.3), phosphoglucomutase (PGM, EC 2.7.5.1), esterase (ES1 and ESD, EC 3.1.1.1), acid phosphatase (ACP, EC 3.1.3.2), β-N-acetylglucosa-

Table 2 Conditions of enzyme staining

Enzyme	Buffer	Substrate	Coenzyme	Other additions
ME	0.1M Tris-HCl, pH 7.0	100mg L-malate (Na)	8mg NADP	8mg MTT, 3mg PMS, 70mg $MgCl_2$, 500mg agar
6PGD	0.1M Tris-HCl, pH 8.0	13mg 6-phosphogluconate (3Na)	7mg NADP	7mg MTT, 5mg PMS, 260mg $MgCl_2$, 500mg agar
SOD	0.1M Tris-HCl, pH 8.0			8mg MTT, 5mg PMS, 500mg agar
HK	0.1M Tris-HCl, pH 7.4	830mg glucose	8mg NADP	10mg MTT, 2mg PMS, 30mg $MgCl_2$, 16mg ATP, 8mg KCN, 500mg agar 10U G6PDH
AK	0.1M Tris-HCl, pH 8.0	24mg adenosine-5′-diphosphate (Na)	12mg NADP	5mg MTT, 5mg PMS, 100mg $MgCl_2$, 100mg glucose, 7U Hexokinase, 500mg agar, 3U G6PDH
PGM	0.1M Tris-HCl, pH 8.0	40mg D-glucose-1-phosphate (Na) with 1% D-glucose-1, 6-diphosphate	8mg NADP	8mg MTT, 3mg PMS, 70mg $MgCl_2$, 500mg agar 8U G6PDH
ES1	0.1M Phosphate buffer, pH 6.5	20mg α-naphthyl acetate in 50% acetone (2ml)		20mg Fast Blue RR
ESD	0.1M Phosphate buffer, pH 6.5	5mg 4-methylumbelliferyl butyrate dissolved in a few drops of acetone		
ACP	0.1M Acetate buffer, pH 4.9	50mg α-naphthyl acid phosphate (Na)		25mg Fast Blue RR 100mg $MnCl_2$
HEX	0.1M Citrate buffer, pH 4.5	2.5mg 4-methylumbelliferyl 2-acetamide-2-deoxy-α-D-glucopyranoside		
MPI	0.1M Tris-HCl, pH 7.5	34mg mannose-6-phosphate (Ba)	12mg NADP	6mg MTT, 4mg PMS, 46mg $MgCl_2$, 500mg agar, 24U GPI, 11U G6PDH
GPI	0.1M Tris-HCl, pH 8.0	26mg D-fructose-6-phosphate (Na)	8mg NADP	8mg MTT, 3mg PMS, 70mg $MgCl_2$, 500mg agar, 8U G6PDH

Each volume of staining mixture except ESD and HEX was 50 ml, while that of ESD and HEX was 5 ml.

Abbreviations: MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide;
PMS phenazine methosulphate; NAD nicotinamide adenine dinucleotide;
NADP nicotinamide adenine dinucleotide phosphate;
ATP adenosine 5'-triphosphate;
G6PDH glucose-6-phosphate dehydrogenase

minidase (HEX, EC 3.2.1.30), mannosephosphate isomerase (MPI, EC 5.3.1.8), and glucosephosphate isomerase (GPI, EC 5.3.1.9).

Soluble protein profiles and 12 zymograms were detected in polyacrylamide gels containing 3 different Ampholines (LKB Produkter) equally, of which the pH ranges were 4.0-6.5, 5.0-8.0, and 3.5-9.5. Each volume of them was 1.2 ml in final gel solution of 60 ml.

The conditions employed for staining of the enzymes are summarized in Table 2. The staining mixtures of 5 enzymes (6PGD, SOD, ESl and ESD, HEX, MPI) were based on the description of Harris and Hopkinson (1976) and HK was stained by the method of Wright *et al.* (1979). Other enzymes (ME, AK, PGM, ACP, GPI) were stained by the method of McManus and Smyth (1979). After electrophoresis the developer solution was applied and the gel was incubated at 37°C until fully developed. Control samples were concurrently run for each enzyme using developer solution without the specific substrate.

The substrates, coenzymes, linking enzymes (hexokinase, glucose-6-phosphate dehydrogenase, glucosephosphate isomerase), MTT and PMS were obtained from Sigma Chemical Co. Reagents for isoelectric focusing were obtained from LKB Produkter.

Results

The species specific characteristics of 3 *Diphyllobothrium* species studied were revealed in soluble protein profiles and isozyme patterns of 2 enzymes (ESl and ESD, ACP) (Figs. 1–4). However, the enzyme polymorphism was observed in the zymograms of ME and HEX in *D. nihonkaiense,* and each of them was different from the isozyme patterns of the other 2 species (Figs. 5–6).

D. nihonkaiense and *Diphyllobothrium* sp. ind. demonstrated the same isozyme patterns of 3 enzymes (6PGD, SOD, HK), which were different from those of *D. latum* (Figs. 7–9). On the other hand *D. latum* and *Diphyllobothrium* sp. ind. indicated the similar MPI isozyme pattern, being different from that of *D. nihonkaiense* (Fig. 10), whereas *D. latum* and *D. nihonkaiense* indicated the similar AK isozyme pattern, being different from that of *Diphyllobothrium* sp. ind. (Fig. 11).

In the zymogram of PGM enzyme polymorphism was observed in each of 3 species, however the isozyme patterns in the pH range from 7.0 to 8.0 revealed the species specific patterns (Fig. 12). Isozyme pattern of GPI was common in 3 species (Fig. 13).

Soluble protein profiles and isozyme pat-

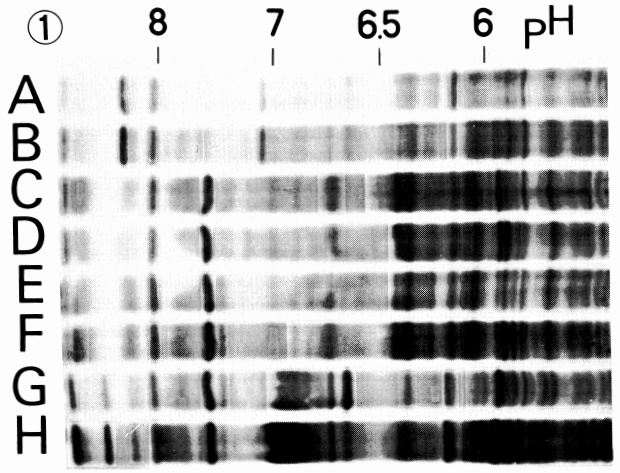
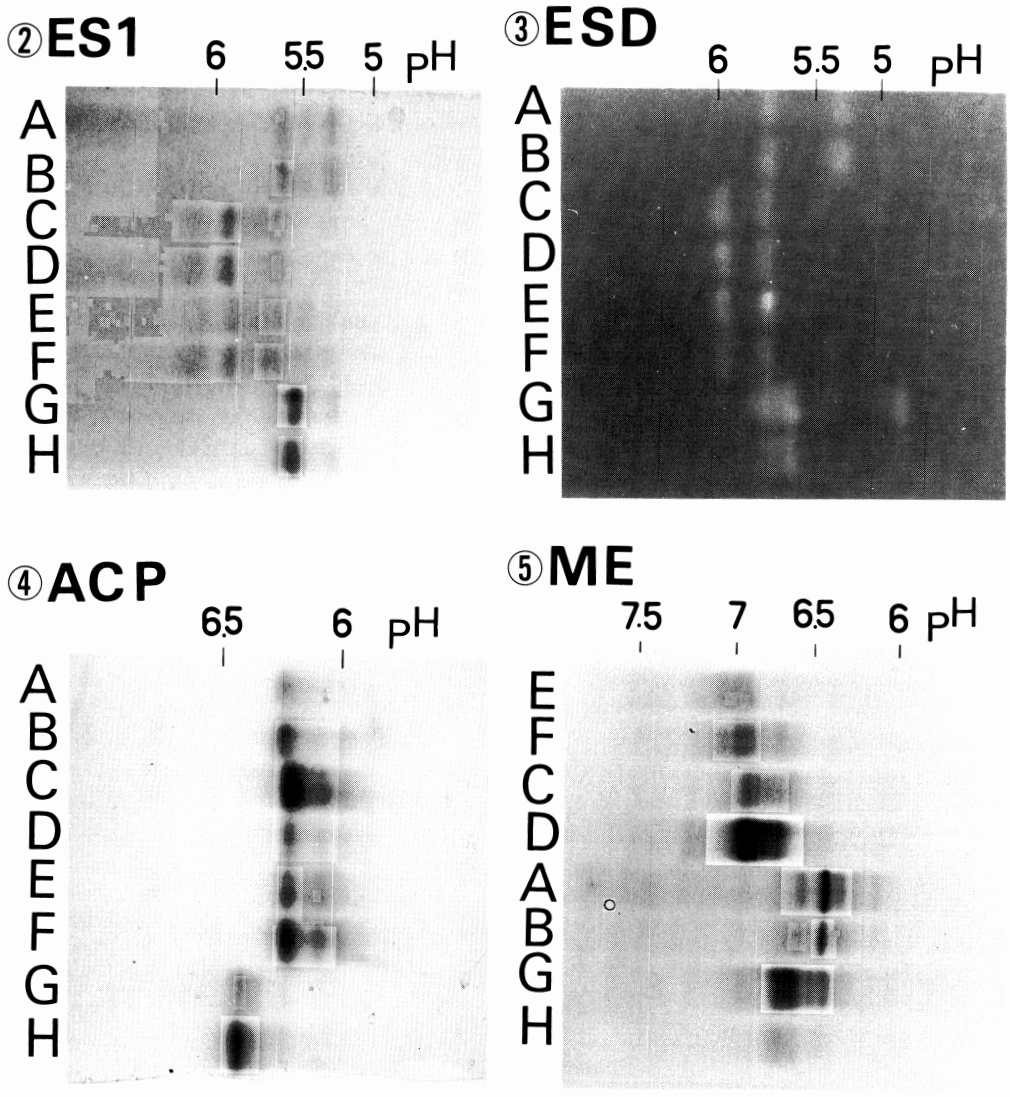


Fig. 1. Soluble protein profiles in 8 specimens of 3 *Diphyllobothrium* species by isoelectric focusing in polyacrylamide gel. A and B: *D. latum*, C–F: *D. nihonkaiense*, G and H: *Diphyllobothrium* sp. ind.

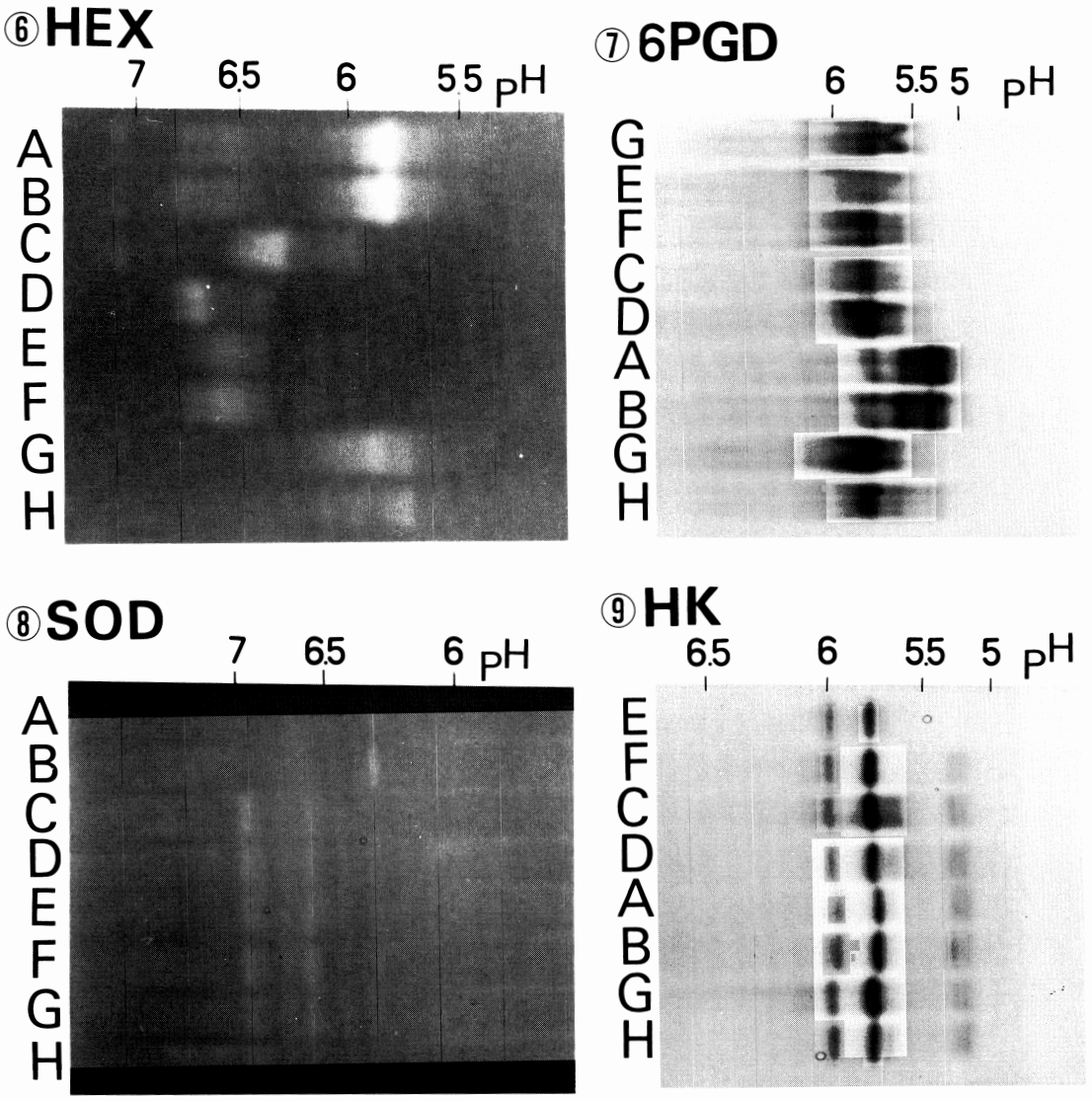


Figs. 2–5. Isozyme patterns in 8 specimens of 3 *Diphyllobothrium* species by isoelectric focusing in polyacrylamide gels. A and B: *D. latum*, C–F: *D. nihonkaiense*, G and H: *Diphyllobothrium* sp. ind.
Fig. 2. ES1: esterase 1
Fig. 3. ESD: esterase D
Fig. 4. ACP: acid phosphatase
Fig. 5. ME: malic enzyme

terns of 8 specimens of 3 *Diphyllobothrium* species were arranged under some categories as shown in Table 3.

Discussion

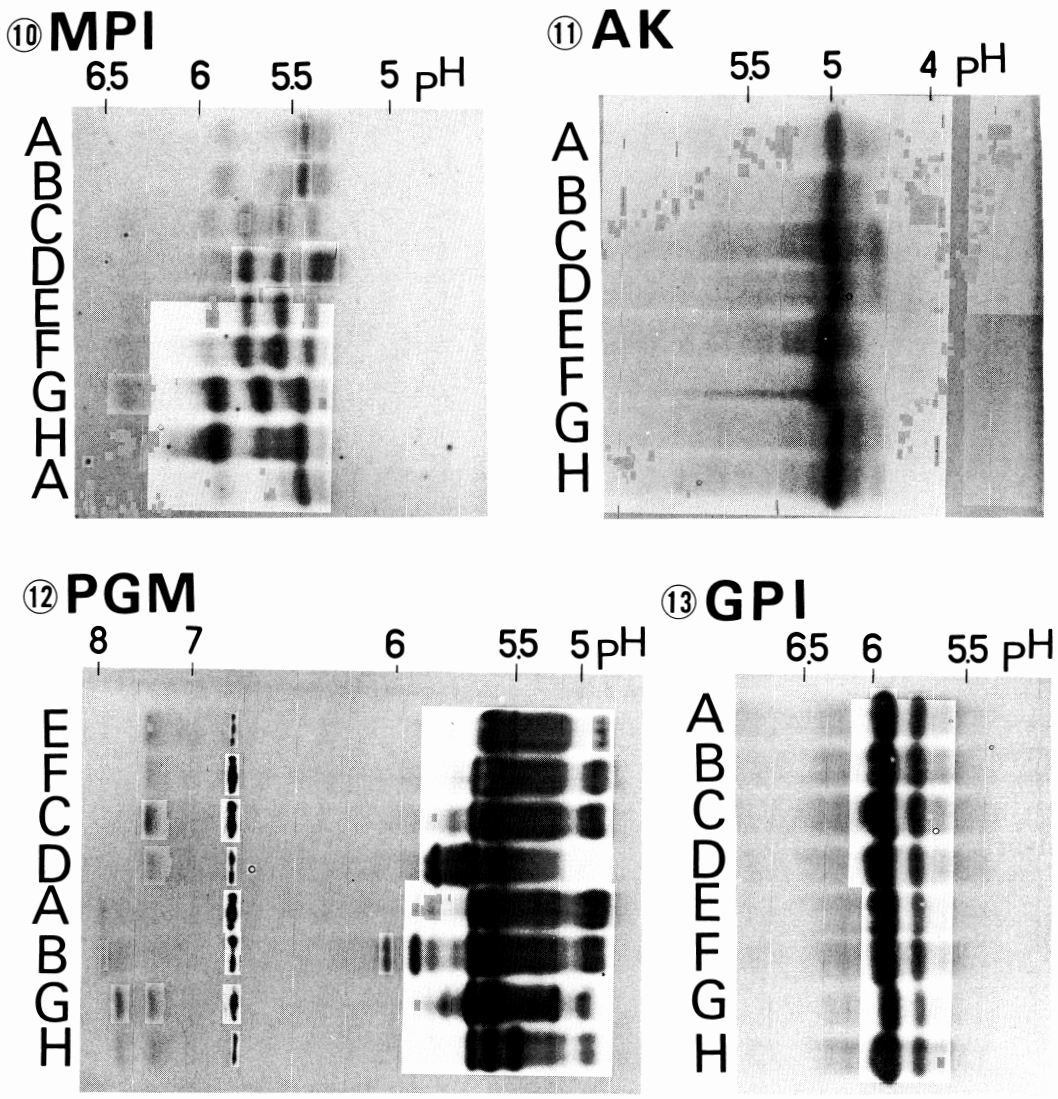
Electrophoretic analysis of tissue protein has been used as a taxonomic tool in some parasitic helminths (Yoshimura, 1968, 1969; Bylund and Djupsund, 1977; Bursey *et al.*, 1980; Kumaratilake and Thompson 1984; Dixon and Arai, 1985: Fukumoto *et al.*, 1987). In the genus of *Diphyllobothrium*, Bylund and Djupsund (1977) reported that 4 species in Northern Europe revealed the species specific protein profiles by isoelectric focusing in polyacylamide gels.



Figs. 6–9. Isozyme patterns in 8 specimens of 3 *Diphyllobothrium* species by isoelectric focusing in polyacrylamide gels. A and B: *D. latum*, C–F: *D. nihonkaiense*, G and H: *Diphyllobothrium* sp. ind.
Fig. 6. HEX: β-N-acetylglucosaminidase
Fig. 7. 6PGD: 6-phosphogluconate dehydrogenase
Fig. 8. SOD: superoxide dismutase
Fig. 9. HK: hexokinase

In the method of Bylund and Djupsund (1977), the concentrates of the supernatants pooled from 12 or 4 specimens per species were used as the samples for electrophoresis, while in the present study the supernatant from each specimen without concentration was used as a sample, and the definite protein bands and zymograms were demonstrated. Therefore, it is possible to compare those of several specimens from the same origin, which seems to be important in order to use the electrophoretic technique for the identification of the species.

In the present study 3 *Diphyllobothrium* species demonstrated the distinct protein bands



Figs. 10–13. Isozyme patterns in 8 specimens of 3 *Diphyllobothrium* species by isoelectric focusing in polyacrylamide gels. A and B: *D. latum*, C–F: *D. nihonkaiense*, G and H: *Diphyllobothrium* sp. ind.
Fig. 10. MPI: mannosephosphate isomerase
Fig. 11. AK: adenylate kinase
Fig. 12. PGM: phosphoglucomutase
Fig. 13. GPI: glucosephosphate isomerase

in the pH range from 6.5 to 8.5. The difference in the protein profiles between *D. latum* and *D. nihonkaiense* was also observed in the pH range from 5.0 to 5.5 (data not shown). Moreover, no variety was observed among the protein profiles from 10 strobilae of *D. nihonkaiense* raised in golden hamsters.

The tapeworm originated from Japanese surfsmelt and olive rainbow smelt was first identified as *Diphyllobothrium ditremum* (Creplin, 1825) by Hotta *et al.* (1978). Later, various differences between *Diphyllobothrium* sp. originated from these fishes and *D. ditremum* from Finland were demonstrated by Yazaki *et*

Table 3 Category arrangement of soluble protein profiles and isozyme patterns in 8 specimens of 3 *Diphyllobothrium* species

	Protein profile	ES1	ESD	ACP	ME	HEX	6PGD	SOD	HK	MPI	AK	PGM (pH 7-8)	PGM (pH 5-6)	GPI
(A) *D. latum*	I	I	I	I	I	I	I	I	I	I	I	I	I	I
(B) *D. latum*	I	I	I	I	I	I	I	I	I	I	I	I	I′	I
(C) *D. nihonkaiense*	II	II	II	II	II	II	II	II	II	II	I	II	II	I
(D) *D. nihonkaiense*	II	II	II	II	II	III	II	II	II	II	I	II	III	I
(E) *D. nihonkaiense*	II	II	II	II	III	IV	II	II	II	II	I	II	II′	I
(F) *D. nihonkaiense*	II	II	II	II	III	IV	II	II	II	II	I	II	II	I
(G) *Diphyllobothrium* sp. ind.	III	III	III	III	IV	V	II	II	II	I′	II	III	II	I
(H) *Diphyllobothrium* sp. ind.	III	III	III	III	IV	V	II	II	II	I′	II	III	IV	I

Each of I' and II' varied a bit in minor bands from other patterns of same category.

al. (1986), in which *Diphyllobothrium* sp. was regarded as a marine species distinguishable from *D. ditremum,* a fresh water species. Bylund and Djupsund (1977) demonstrated as one of the electrophoretic characteristics in *D. ditremum* that proteins at pH 6.50–7.00 were sparse, while in our study the distinct protein bands at the same pH range were recognized in *Diphyllobothrium* sp. originated from Japanese surfsmelt. This result is coincident with the conclusion of Yazaki *et al.* (1986).

Soluble protein profiles were of great advantage to compare many loci in a single gel, but the property of proteins focused in each band might not be identified. Therefore it is difficult to consider that allelic diversity of 3 *Diphyllobothrium* species were exactly estimated. By contrast, it might be possible to speculate on the genetic similarity and variability among 3 *Diphyllobothrium* species on the basis of the data from isozyme analysis.

In the isozyme patterns of 2 enzymes (ESl and ESD, ACP) 3 *Diphyllobothrium* species demonstrated the species specific patterns. Although enzyme polymorphism of ME and HEX was observed in *D. nihonkaiense,* each of these isozyme patterns was different from either pattern of *D. latum* or *Diphyllobothrium* sp. ind. The isozyme pattern of GPI was reported to be useful in the differentiation of taeniid cestodes (Le Riche and Sewell, 1977, 1978b) and *Echinococcus* strains or species (Le Riche and Sewell, 1978a; McManus and Smyth, 1979; Macpherson and McManus, 1982), while 3 *Diphyllobothrium* species examined in the present study revealed the common isozyme pattern of GPI.

Judging from the isozyme profiles of other 5 enzymes (SOD, 6PGD, HK, MPI, AK), the genetic relation between *D. latum* and *D. nihonkaiense* supposed to be more distant than that between *D. nihonkaiense* and *Diphyllobothrium* sp. ind.

The results described here are based on small number of specimens, and more fresh specimens must be examined to evaluate the extent of enzyme polymorphism within each species. In any case, the results of this study supports the proposal by Yamane *et al.* (1986) that *D. nihonkaiense* is a distinct species differentiated from *D. latum* (Linné, 1758).

Fukumoto (1985) reported that PK isozymes in *Spirometra erinacei* changed with parts of strobilae. In the present study the specimens from 2 different parts of the strobila (immature proglottids with scolex and mature proglottids with immatures) raised in golden hamster demonstrated the coincident isozyme patterns.

Two specimens of *D. latum* expelled from the same host at 4 years intervals indicated the same isozyme patterns and the same soluble protein profile, which gave the evidence that the enzymatic activities of *D. latum* were hardly lost after 4 years storage at –80°C.

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