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Interspecific Hybridization in Three Species, *Paragonimus ohirai, P. iloktsuenensis* and *P. sadoensis*, with Special Reference to Isozyme Patterns in F_1 Hybrids

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Introduction

The adult morphology in three species of lung flukes, *Paragonimus ohirai, P. iloktsuenensis*, and *P. sadoensis*, is too similar to distinguish one from another, though many morphological studies have been made (Chen, 1940; Isshiki, 1953; Komiya *et al.*, 1960; Tomimura, 1959 a, b, c; Miyazaki, 1945). On the other hand, they are easily distinguishable by their metacercarial characteristics; that is, the metacercariae of *P. ohirai* and *P. sadoensis* have two membranes, outer and inner, while that of *P. iloktsuenensis* lacks the inner membrane. Moreover, the larval body size of *P. ohirai* and *P. sadoensis* is significantly larger than that of *P. iloktsuenensis*. The main differences between *P. ohirai* and *P. sadoensis* are in the shape and color of the metacercaria (Kawashima, *et al.*, 1967). We conducted a series of population genetical studies of the isozymes in these three species, and found that there was a high genetic similarity among them (Agatsuma and Habe, 1985 a). Furthermore, we discovered that even differences in metacercarial characteristics, which heretofore have been regarded as most important for specific discrimination, seem to be only hereditary phenomena within the same species (Habe, *et al.*, 1985). This indicates that the three species should be categorized as a single species. In order to confirm hybrid formation in F_1 progeny among these three species, cross experiments between two out of the three were conducted using enzyme electrophoretic variants. In this communication, we report interspecific F_1 hybrid isozyme patterns of two enzymes, phosphoglucomutase (PGM : EC 2.7.5.1) and glutamic-oxaloacetic transaminase (GOT : EC 2.6.1.1), and demonstrate that hybridization does occur in all combinations of the cross experiments.

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Materials and Methods

Metacercariae of *P. ohirai* and *P. iloktsuenensis* were harvested from the crab, *Sesarma dehaani*, collected in Kinosaki, Hyogo Prefecture and Amami Island, Kagoshima Prefecture, in Japan, respectively. *P. sadoensis* metacercariae were, on the other hand, harvested from the crab, *Geothelphusa dehaani*, on Sado Island, Niigata Prefecture, in Japan. Three kinds of cross combinations were made: *P. ohirai* × *P. iloktsuenensis*, *P. ohirai* × *P. sadoensis*, and *P. iloktsuenensis* × *P. sadoensis*. In each cross, two of the metacercariae, each of which is derived from a different species, were introduced intraperitoneally into each albino rat (Wistar). Sixty days after inoculation adult worms were recovered from the rat lung cysts. The eggs from each worm were separately incubated at 28°C for twenty five days. Miracidia hatched from the eggs were then exposed to brackish water snails, *Angustassiminea parasitologica*, which were collected from Fukuoka, where no infected crab has been found so far. Cer-

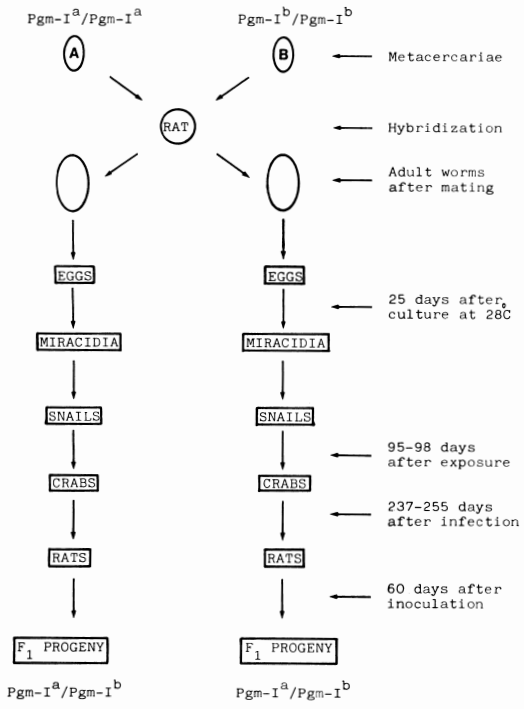

Fig. 1 Diagram of the procedure used in performing interspecific hybridization and the result of F_1 hybrid genotypes of an enzyme, phosphoglucomutase (EC 2. 7. 5. 1), as an example, in a cross between *Paragonimus ohirai* and *P. iloktsuenensis*.

cariae were observed in snail hosts 95-98 days after infection. The cercariae derived from each parent were separately introduced into brackish crabs, *Sesarma dehaani*, sampled from Fukuoka, where no infected crab has been observed. Metacercariae were recovered from the crab host 237-255 days later. Each parental group of metacercariae was separately inoculated into a rat. Adult worms obtained from the rat 60 days after inoculation were examined electrophoretically. Fig. 1 shows a diagram of the cross procedure between different species. All adult samples were washed and then stocked in a deep freezer at −80°C until required for electrophoresis. The extracts were prepared by homogenizing each, individually with 100 μl of 0.1 M phosphate buffer solution (pH 7.5) using a Teflon homogenizer in an ice water bath. The homogenized worms were centrifuged at 3,000 rpm for 3 min at room temperature, and the supernatants were used in this study. The amount of starch used was 12.0 g/ml of gel buffer. Buffer systems and staining mixtures for two enzymes are as follows (Shaw and Prasad, 1970).

1) *Phosphoglucomutase* (PGM)

Bridge buffer(pH 6.0) : 0.378 M Tris–0.165 M citric acid. Gel buffer : a one–30 th dilution for bridge buffer. Staining mixture : glucose-1-phosphate 50 mg, NADP 2 mg, MTT 2 mg, PMS 2 mg, G6PD 2 U, G-1,6-P trace. Electrophoresis was run for 5 hr at a constant current of 60 mA.

2) *Glutamic-oxaloacetic transaminase* (GOT)

Bridge buffer (pH 8.2) : 0.3 M boric acid-0.06 M NaOH. Gel buffer (pH 8.7) : 0.076 M Tris-0.005 M citric acid. Staining mixture : L-aspartic acid 200 mg, α-ketoglutaric acid 100 mg, Fast blue BB 100 mg, EDTA 200 mg. Electrophoresis was run for 5 hr at a constant voltage of 250 V.

Results

1. Cross experiment between *P. ohirai* and *P. iloktsuenensis*

Only phosphoglucomutase was examined in F_1 hybrid progeny. We have already found that *Paragonimus* PGM is controlled by two loci, *Pgm*-I and *Pgm*-II, and that there are two alleles *Pgm*-I^a and *Pgm*-I^b at *Pgm*-I locus in natural populations of both species (Agatsuma and Habe, 1985 a). Moreover, we found that a homozygote, *Pgm*-I^a/*Pgm*-I^a, is predominant in the Kinosaki population of *P. ohirai*, while another homozygote, *Pgm*-I^b/*Pgm*-I^b, is predominant in the Amami population of *P. iloktsuenensis* (Agatsuma and Habe, 1985 a). Two F_1 progeny groups from each parent were examined ; the number of F_1 individuals examined was 22 for one parent, and 15 for the other. All phenotypes of PGM in the progeny were a two-banded pattern, showing a heterozygote, *Pgm*-I^a/*Pgm*-I^b, which is the same as that occasionally seen in natural populations of both species (Fig. 2).

2. Cross experiment between *P. ohirai*

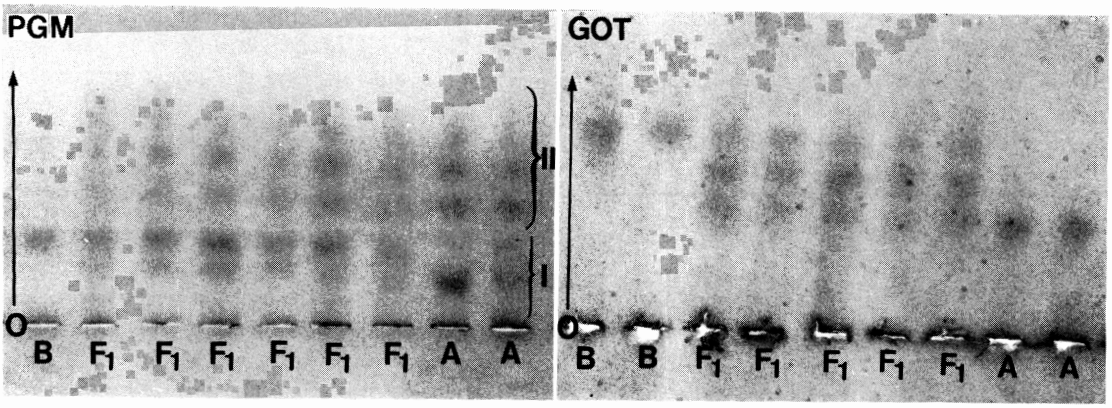

Fig. 2 Photographs of electrophoretic phenotypes observed in F_1 hybrid progeny between *P. ohirai* and *P. iloktsuenensis* for PGM, and between *P. ohirai* and *P. sadoensis* for GOT.
PGM……A : *P. iloktsuenensis* from Amami Island, B : *P. ohirai* from Kinosaki (I and II show the locus number responsible for phosphoglucomutase phenotype. See text for details.).
GOT……A : *P. ohirai* from Kinosaki, B : *P. sadoensis* from Sado Island
O : origin of the sample slot, F_1 : hybrid progeny in F_1 generation

and *P. sdoensis*

a) *Phosphoglucomutase (PGM)*

P. sadoensis PGM phenotype distribution is quite unique. We found this locus monomorphic, showing the existence of only a single allele, which was identified as *Pgm*-I^b (Agatsuma and Habe, 1985 a). Fifteen individuals of F_1 progeny derived from only one parent were examined, and it was found that all individuals examined had a two-banded phenotype, showing a heterozygote, *Pgm*-I^a/*Pgm*-I^b, which is the same as that observed in the F_1 from the cross experiment 1 (*P. ohirai* × *P. iloktsuenensis*) (Fig. 2).

b) *Glutamic-oxaloacetic transaminase (GOT)*

There are three alleles, Got^a, Got^b and Got^c, in natural populations of *P. ohirai*, and two alleles, Got^a and Got^b, in *P. sadoensis*, but it has been found that Got^a/Got^a is predominant in the Kinosaki population of *P. ohirai*, and Got^b/Got^b, in the Sado population of *P. sadoensis* (Agatsuma and Habe, 1985 a). In this cross, all F_1 progeny (15 individuals), which were identical to those examined for the PGM system, were also used for GOT isozyme inheritance, and it was found that all individuals possessed a triplet band, indicating a heterozygote, Got^a/Got^b (Fig. 2). The triplet band is the same as those seen in natural populations of *P. ohirai* (Agatsuma and Habe, 1985 a).

3. Cross experiment between *P. iloktsuenensis* and *P. sadoensis*

a) *PGM*

As described above, *Pgm*-I^b/*Pfm*-I^b is predominant in both species. All F_1 progeny (16 individuals) from this cross showed only a single band as expected, indicating a homozygote, *Pgm*-I^b/*Pgm*-I^b (Fig. 2).

b) *GOT*

The same F_1 progeny was also examined for this enzyme system. All F_1 individuals examined (16 individuals) were found to have a triplet band, which was identical to those observed in F_1 progeny from the cross experiment 2 (*P. ohirai* × *P. sadoensis*) (Fig. 2), indicating a heterozygote, Got^a/Got^b.

Discussion

Enzymes are the products of genes. Variations in enzymes are thus a direct reflection of genetic variation. Most enzymes are under the control of nuclear genes which undergo a straight-forward Mendelian pattern of inheritance. This indicates that enzyme variants are of particular value in genetic work including inter-specific hybrid studies. In parasitic protozoa, many studies on genetic cross

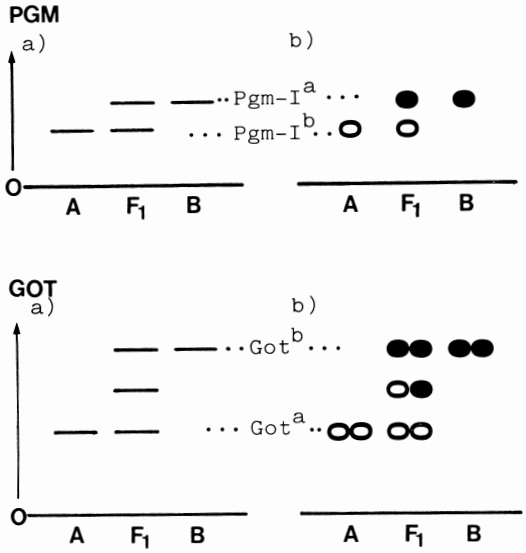

Fig. 3 a) Schematic presentation of PGM and GOT isozyme phenotypes observed in F_1 hybrid progeny and individual adults (A and B) from natural populations of the *Paragonimus* species. A and B are the same as those in Fig. 2. b) Diagrammatic representation of molecular combination in the two enzyme patterns in *Paragonimus* species. Symbols: PGM··· ● shows a protein produced by the *Pgm*-I^a allele; ○ shows a protein produced by the *Pgm*-I^b allele
GOT····· ● shows a subunit produced by the *Got*^{b} allele; ○ shows a subunit produced by the *Got*^{a} allele. For the other abbreviations, see the legend of Fig. 2

experiments have been reported using isozymes as markers, especially in relation to the recombination of drug resistant genes (e.g. Walliker, 1983). However, only a few have been reported on intra- or inter-specific hybridization experiments using enzyme variants (isozymes) in the parasitic helminth (Wright and Southgate, 1976; Wright and Ross, 1980). Wright and Ross (1980), using two enzyme systems, G6PD and PGM, carried out a cross experiment between *Schistosoma haematobium* and *S. matthei*, and found their F_1 hybrid enzymes to be additive, being a combination of those of the two parental species. They concluded that hybridization does occur between the species. Recently, we performed an intra-specific cross experiment between GOT variants of *P. ohirai* from Kinosaki, Hyogo Prefecture, and found that the variants are controlled by two codominant alleles at a single locus whose products aggregate at random, forming a dimer (Agatsuma and Habe, 1985 b).

In the present study we performed some cross experiments between different species of *Paragonimus*, and examined two enzyme systems, phosphoglucomutase (PGM) and glutamic-oxaloacetic transaminase (GOT), of the F_1 hybrids using starch gel electrophoresis. We found that all F_1 hybrid PGM possessed two bands, each of which was derived from the parental species. This two-banded pattern was the same as the heterozygote which was produced in the intra-specific cross between the PGM variants in *P. ohirai* (Agatsuma and Habe, unpublished data). In the case of GOT, all F_1 progeny similarly showed a triplet band pattern, having an additional hybrid molecule between the two parental bands. This triplet band pattern was also the same as the heterozygote which was produced in the intra-specific cross between the GOT variants in *P. ohirai* (Agatsuma and Habe, 1985 b). This indicates that the genetics of PGM and GOT isozymes is the same in the three species. Fig. 3 shows a diagrammatic representation of molecular combinations in the GOT dimer enzymes in F_1 hybrids, compared with the monomer type of the enzyme, PGM, in F_1 hybrids. The present result shows that inter-specific hybridization occurs experimentally between these species, and clearly explains the previous findings in which genetic distance between the sympatric species of *P. ohirai* and *P. iloktsuenensis* from Sendai, Kagoshima Prefecture, was far smaller than even the distance between intraspecific populations in *P. ohirai* or *P. iloktsuenensis* (Agatsuma and Habe, 1985 a). We can now interpret these facts as a gene flow, or mating, occurring naturally between the two species. At any rate, the present results support the previous conclusion that these three species should be categorized as a single species.

Summary

Three cross experiments were carried out between the different *Paragonimus* species, *P. ohirai*, *P. iloktsuenensis* and *P. sadoensis*, and, using two enzyme systems, phosphoglucomutase (PGM) and glutamic-oxaloacetic transaminase (GOT), their F_1 hybrids were examined by starch gel electrophoresis. In the cross between *P. ohirai* and *P. iloktsueensis*, all the F_1 hybrid PGM (37 individuals) showed two bands, each of which is derived from the parental species, showing a heterozygote, $Pgm\text{-}I^a/Pgm\text{-}I^b$. In the case of the cross between *P. ohirai* and *P. sadoensis*, all F_1 fifteen individuals examined had a two-banded phenotype of PGM, which is the same as those observed in the cross between *P. ohirai* and *P. iloktsuenensis*. On the other hand, for the GOT system, all F_1 (15 individuals) possessed a triplet band, indicating a dimer molecule, which had an additional hybrid molecule lying between the two parental bands. The cross, *P. iloktsuenensis* × *P. sadoensis*, produced only heterozygous GOT (Got^a/Got^b) F_1 progeny (16 individuals). These results indicate that inter-specific hybridization occurs experimentally between these species, and support our previous conclusion that these three species may be the same species.

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3種肺吸虫 (*Paragonimus ohirai, P. iloktsuenensis* 及び *P. sadoensis*) における種間雑種：F_1 雑種個体におけるアイソザイムパターン

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3種類の種間交配実験，すなわち，城崎産大平肺吸虫 (*P. ohirai*：PO_K)×奄美産小型大平肺吸虫 (*P. iloktsuenensis*：PI_A)，城崎産大平肺吸虫×佐渡産佐渡肺吸虫 (*P. sadoensis*：PS_S) 及び奄美産小型大平肺吸虫×佐渡産佐渡肺吸虫，を行い，それらの F_1 雑種個体の2種の酵素，phosphoglucomutase (PGM：EC 2.7.5.1) 及び glutamic-oxaloacetic transaminase (GOT：EC 2.6.1.1) の泳動パターンをデンプンゲル電気泳動により調べた．

まず，交配実験，$PO_K \times PI_A$，において，その PGM のパターンを調べてみると，調査したすべての F_I 雑種個体 (37個体) が2本のバンドをもっており各バンドは，それぞれ異種親由来のものであることがわかった．また $PO_K \times PS_S$ において，まず PGM パターンを調べてみると，F_I 個体すべて (15個体) が2本のバンドを有しており，この2本の各バンドは，$PO_K \times PI_A$ 交配の F_I 雑種個体同様，それぞれ異種親由来のものであった．一方，GOT パターンでは，F_1 (15個体) は，すべて3本のバンドを有しており，これは，異種親由来の2本のバンドに，その雑種分子のバンドが加わったもので，dimer 構造をもつ酵素のヘテロ接合体を示すものであった．さらに，$PI_A \times PS_S$ においても，その F_I 子孫 (16個体) の GOT パターンは，すべて3本であり，$PO_K \times PS_A$ の F_1 の GOT パターン同様，ヘテロ接合体を示すことがわかり，異種由来の遺伝子が導入されたことがわかった．これらの一連の交配実験の結果は，すべての交配において，種間雑種が形成されたことを証明するものであり，先に得られた結論すなわちこれらの3種は同一種の可能性があるとの結論 (Agatsuma and Habe, 1985 a; Habe *et al.*, 1985) を支持するものである．