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Mitochondrial DNA differentiation of Japanese diploid and triploid *Paragonimus westermani*

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Abstract

Restriction endonuclease analysis of mitochondrial DNA was carried out in order to differentiate diploid and triploid types of *Paragonimus westermani* originating from Japan. It was found that out of 16 restriction enzymes. PstI, HaeIII and RsaI revealed consistently different cleavage patterns between the two types.

Introduction

Japanese diploid and triploid populations of *Paragonimus westermani* have been well analysed from a viewpoint of isozyme and chromosome studies (Agatsuma & Habe, 1985; Hirai & Agatsuma, 1991; Hirai *et al.*, 1992). From the isozyme study, it has been demonstrated that five triploid enzyme loci contained triploid fixed-alleles which are hardly seen, or absent from Japanese diploid populations examined so far, suggesting that those alleles originated from non-Japanese flukes (Hirai & Agatsuma, 1991).

Analysis of mitochondrial DNA (mt DNA) can provide unique insights into the history of triploid origin, because of its properties of maternal inheritance and rapid evolution (Attardi, 1985). In the present study, the restriction fragment length polymorphism (RFLP) analysis of mt DNA was carried out in order to find other genetic markers that differentiate between the Japanese diploid and triploid *P. westermani*.

Materials and methods

Paragonimus *samples*

Diploid metacercariae were obtained from three localities, Ohita (5), Mie (5), and Chiba (5) in Japan, and triploid ones from four localities, Tsushima (5), Amakusa (4) and Yakushima (2) in Japan, and Bogil Island (5) in Korea (examined adult worm numbers in parenthesis). The metacercariae obtained were fed to dogs and cats, and adults were recovered after about six months. Adult samples were kept at −80°C until used.

Mt DNA preparation

Mitochondrial DNA (mt DNA) was prepared according to the method of Tamura & Aotsuka (1988) with slight modifications. Frozen adult flukes were individually homogenized in 1 ml of a chilled homogenizing buffer (0.25 mM sucrose, 10 mM EDTA, and 30 mM Tris-HCl, pH 7.5) using a teflon homogenizer. The homogenate was centrifuged at 1000 g for 3 min at 4°C to remove the nuclei and cell debris. The supernatant recovered was recentrifuged at 12,500 g for 10 min at 4°C to pellet mitochondria. The mitochondrial pellet was suspended in 10 mM Tris-EDTA buffer (0.15 M NaCl and 10 mM EDTA, pH 8.0), and 0.18 N NaOH containing 1% sodium dodecyl sulfate (SDS) and subsequently 3 M potassium and 5 M acetate solution were added to the pellet suspension. The suspension mixture was centrifuged at 12,000 g for 5 min at 4°C and the supernatant was treated with phenol/chloroform twice. The solution was extracted once more with chloroform, the mt DNA precipitated with ethanol, and then resuspended in an appropriate volume of sterile distilled water. The mt DNA prepared by this method was of sufficient purity for restriction enzyme analysis.

Restriction endonucleases

Partially purified mt DNAs were digested with 16 restriction enzymes; 12 six-base cutting enzymes; AccI, ApaI, BglII, EcoRV, HincII, PstI, PvuII, SacI, SalI, SmaI, SphI and XbaI, 4 four-base cutting enzymes; HaeIII, HinfI, MspI and RsaI.

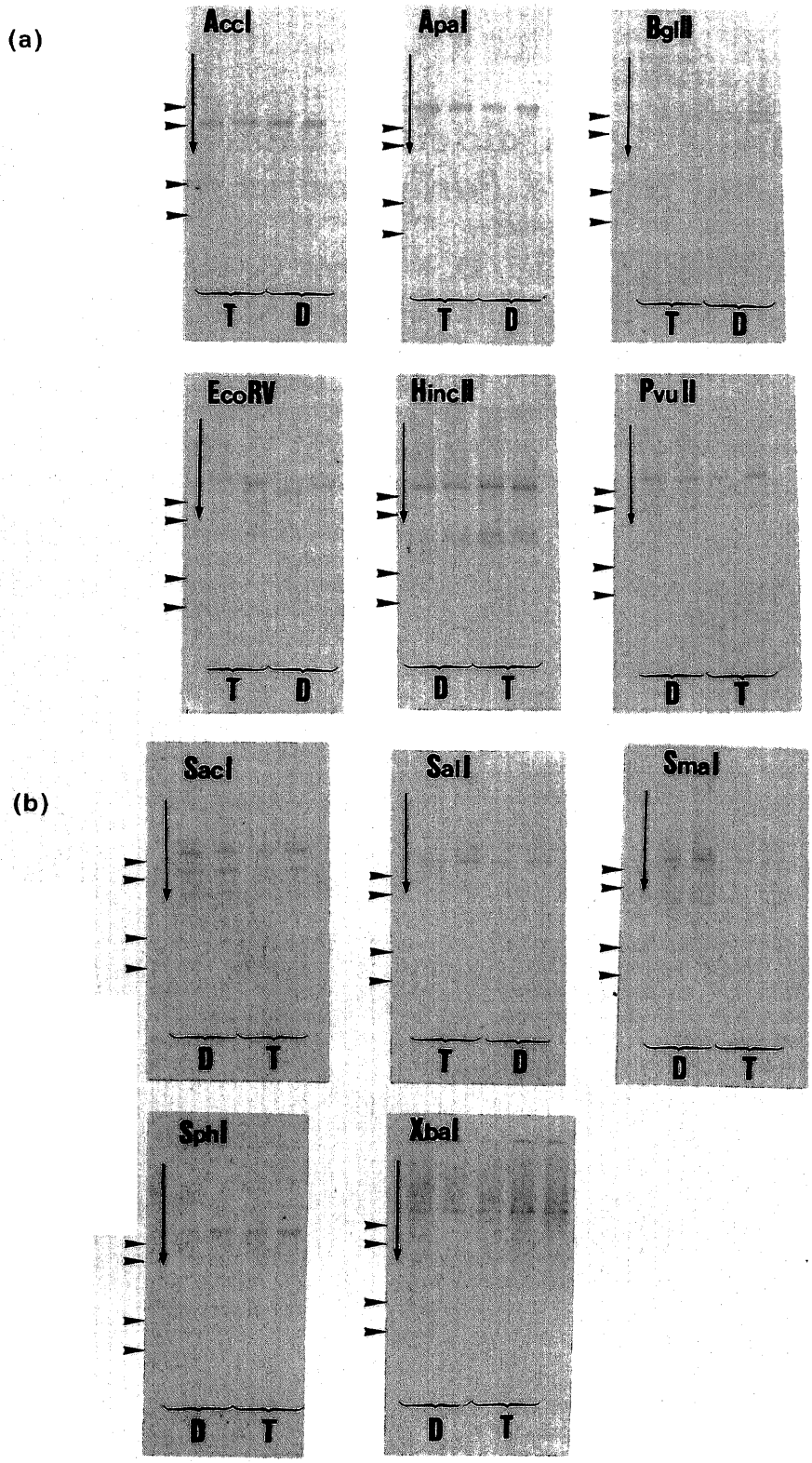

Fig. 1. Southern blot of endonuclease cleavage patterns in mt DNA obtained from diploid (D) and triploid (T) *P. westermani*, and hybridized with dig-dUTP-labelled probe. a) AccI, ApaI, BglII, EcoRV, HincII and PvuII, b) SacI, SalI, SmaI, SphI and XbaI. From right to left; Amakusa (A), Yakushima (Y), Ohita (O) and Mie (M) for each endonuclease cleavage pattern. Arrows show 10.0 Kbp, 5.0 Kbp, 1.0 Kbp and 0.5 Kbp from the top.

Electrophoresis

Digested DNA samples were size fractionated on 1% (w/v) agarose gel for six-base cutting enzymes and stained with ethidium bromide. Gradient polyacrylamide gel (4–15%) was conducted for four-base cutting enzymes and silver-stained as per manufacturer's instructions (Wako).

Southern blotting and hybridization

After agarose electrophoresis, the gels were blotted onto Hybond N nylon membranes using the method of Southern (1975). Partially purified mt DNA originating from a triploid fluke (Tsushima) was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate using a Dig-DNA labelling and detection kit obtained from Boehringer Mannheim as per manufacturer's instructions. Filters were prehybridized at 65°C for 2–3 hours in 5×SSPE (20×SSPE is 3.6 M NaCl; 0.2 M sodium citrate; 0.02 M EDTA, pH 7.7); 5×Denhardt's solution; 0.5% SDS; and 100 µg/ml sheared denatured herring sperm DNA. Hybridization took place overnight at 65°C with a probe concentration of approximately 50 ng of labelled DNA per ml hybridization solution. The filters were washed at 65°C with 2×15 min washes with 2×SSPE and 0.1% SDS, 1×10 min wash with 1×SSPE and 0.1% SDS, and 1×10 min wash with 0.1×SSPE and 0.1% SDS. The filters were then used to carry out immunological detection according to the manufacturer's instructions (Boehringer Mannheim).

Results and discussion

For six-base cutting enzymes, no variations were found between or within types in the eleven enzymes; AccI, ApaI, BglII, HincII, PvuII, SacI, SalI, SmaI, SphI and XbaI (fig. 1a, b). Four enzymes, ApaI, SalI, SphI and XbaI exhibited a single band, indicating only one cutting site. The other enzymes were found to have two cutting sites (BglII, EcoRV, PvuII and SmaI), three (AccI; double bands of approximately 10 Kbp fragment plus 1 Kbp fragment, and SacI), and six (HincII). Variation was observed from a diploid population

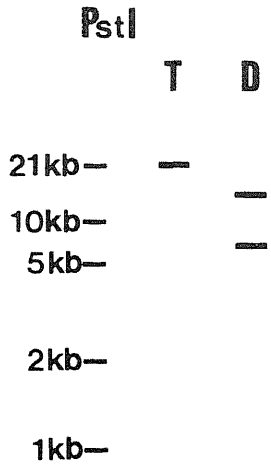

Fig. 2. Ethidium bromide-stained agarose gel (1%) showing Pst I cleavage patterns of mt DNA obtained from diploid (D) (Amakusa) and triploid (T) (Mie) *P. westermani*.

(Mie) in EcoRV cleavage pattern. On the other hand, PstI pattern was distinct between the two types, as shown in fig. 2 which shows representative patterns of the two types (triploid, Amakusa and diploid, Mie). The figure indicates that the triploid lost one cutting site. Cleavage site numbers in 12 restriction enzymes are shown in table 1. The whole length of the mt DNA is estimated to be approximately 21 kbp.

For four-base cutting enzymes, variations in HinfI and MspI patterns were found and shared among the two types (see arrows). On the other hand, HaeIII and RsaI showed distinct type-specific differences in bands indicated by arrows, though there are also minor variations within each type (fig. 3).

Wang *et al.* (1991) carried out comparative studies on repetitive DNA RFLP patterns between the two types of *P. westermani*, using Southern blotting hybridization

Table 1. Numbers of cleavage sites of *Paragonimus westermani* mt DNA for 12 restriction endonuclease and the molecular weights of their fragments in kilo base pairs (kbp)

Restriction endonuclease	Type of cleavage patterns	Number of cleavage sites	Molecular weights of fragments (kbp)		
AccI	A	3	10.1	9.9	1.0
ApaI	A	1	21.0		
BglII	A	2	11.0	10.0	
EcoRV	A	2	17.5	3.5	
	B	6	16.5	1.5	1.4
			0.7	0.6	0.3
HincII	A	6	13.0	3.5	2.5
			1.0	0.7	0.3
PstI	A	2	14.5	6.5	
	B	1	21.0		
PvuII	A	2	15.8	5.2	
SacI	A	3	12.0	5.5	3.5
SalI	A	1	21.0		
SmaI	A	2	17.5	3.5	
SphI	A	1	21.0		
XbaI	A	1	21.0		

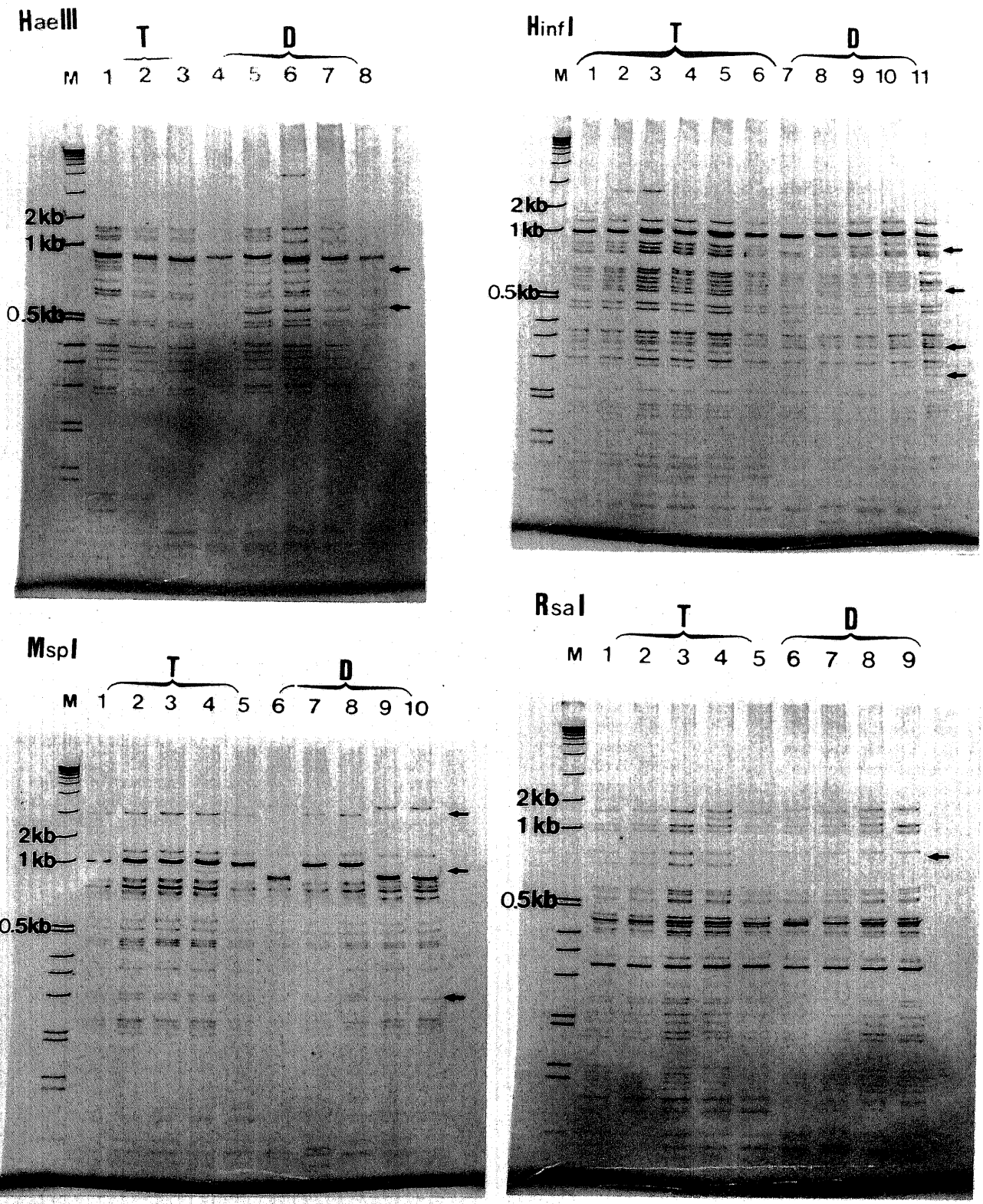

Fig. 3. Silver stained-polyacrylamide gel (4–15%) showing cleavage patterns of four base cutting-endonuclease (HaeIII, HinfI, MspI and RsaI) in mt DNA obtained from diploid (D) and triploid (T) *P. westermani*. Arrows show variations within and/or between the two types. HaeIII: 1;K, 2;A, 3;T, 4;H, 5;H, 6;M, 7;C, 8;M HinfI: 1;K, 2;K, 3;A, 4;A, 5;A, 6;T, 7;M, 8;O, 9;O, 10;C, 11;C MspI: 1;K, 2;A, 3;A, 4;A, 5;T, 6;M, 7;O, 8;O, 9;C, 10;C RsaI: 1;K, 2;K, 3;A, 4;A, 5;T, 6;M, 7;O, 8;C, 9;C A: Amakusa, C: Chiba, K: Korea (Bogil Island), M: Mie, O: Ohita, T: Tsushima

techniques of total genomic DNA. They demonstrated that the RFLP patterns of four restriction enzymes. DdeI, HaeIII, HpaII and PstI were type-specific, supporting our previous hypothesis.

In the present preliminary study with two restriction endonucleases (PstI and HaeIII), digestion patterns were found to differentiate the triploids from the diploids. MspI (an isoschizomer of HpaII) gave similar results, but the Ohita diploid population was shown to be identical to a typical triploid cleavage pattern as seen in fig. 3. In this study, another new enzyme (RsaI) RFLP pattern was found to be diagnostic in the type differentiation. These results again strongly support our hypothesis that the triploids may have arisen from hybridization between strains of a Japanese and a non-Japanese diploid of *P. westermani* (Agatsuma & Habe, 1985; Hirai & Agatsuma, 1991). Restriction-endonuclease analysis of mt DNA is now widely used for evolutionary population genetic studies. In particular, the lack of recombination and the maternal mode of inheritance would become favoured properties for the study of gene introgression involved in the hybridization event to create a new race, such as a triploid. If the Japanese triploid was of hybridization origin, the Japanese diploid may have given the paternal role, because our isozyme data strongly suggested that a certain individual of the Japanese diploid must have been involved in the assumed hybridization event (Agatsuma & Habe, 1985). Besides, the low level of heterogeneity within the triploid populations implies that the triploid may be of recent origin.

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