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Proposals for a new classification of iridescent viruses

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The need for comparative studies of iridoviruses to elucidate the relationships between them has been well appreciated. Sixteen iridoviruses, including type species from each of the four recognized genera of the Iridoviridae, were compared by restriction endonuclease characterization, hybridization to the major structural protein (MSP) gene of an invertebrate iridescent virus (IV) isolate at various stringencies, PCR amplification of the MSP gene region and by dot-blot hybridization studies. The results broadly supported previous serological studies. The vertebrate iridoviruses, frog virus 3 (genus *Ranavirus*) and flounder lymphocystivirus (genus *Lymphocystivirus*), appeared distinct from one another and from the invertebrate isolates. Naming and numbering invertebrate IV isolates according to history and host is no longer useful since IVs infect a number of species. A revised system, involving names based on the geographical origin of the isolate is proposed, in line with other virus families. The large IVs of invertebrates represented by Vero Beach IV (previously IV3 or mosquito IV; genus *Chloriridovirus*) showed little similarity to any other IVs. Members of the genus *Iridovirus*, the small invertebrate IVs, fell into three distinct groups of interrelated isolates. The largest group, containing the Plowden (IV1), Tia (IV2), Nelson (IV9, IV10 and IV18), Aberystwyth (IV22), Srinagar (IV24), Fort Collins (IV29) and Stoneville (IV30) iridoviruses, is named the Polyiridovirus complex. The Plowden iridovirus (IV1) is suggested as type species for this complex given the data available on its molecular biology. Based on previously published data, Timaru (IV16 and IV19) and Uitenhage (IV23) iridoviruses are also assigned to this complex. The second but smaller group is named the Oligoiridovirus complex, which includes Dazaifu (IV6) as the type species and contains Ntondwe (IV21 and IV28) on a tentative basis. Riverside IV (IV31) was distinct from both of the other groups, and is proposed as a third complex, Crustaceoiridovirus.

Introduction

The most informative schemes of classification aim to group each assemblage systematically according to shared ancestries and phylogenetic interrelationships; common physical, biochemical and genetic factors are used to infer evolutionary histories. Iridoviruses are non-occluded icosahedral particles, containing a large dsDNA genome of between 100 and 200 kbp. The viruses assemble in host cytoplasm. Iridoviruses have been isolated from both vertebrate and invertebrate hosts. Invertebrate iridescent viruses (IVs) have been classified by host and number, according to the sequence in which they were described (Tinsley & Kelly, 1970). Thus, the first IV to be discovered was named *Tipula paludosa* iridescent virus (or IV1), the second, *Sericesthis pruinosa* iridescent virus (or IV2), and so on. This system takes no account of phylogenetic associations, mainly owing to the lack of knowledge concerning the relationships among these viruses. Within the invertebrate IVs two genera are recognized. Members of the genus *Chloriridovirus* (particles of around 180 nm in diameter; type species mosquito IV, IV3), have only been described from Diptera. Members of the genus *Iridovirus*, which have particles of some 130 nm in diameter, have been isolated from a broader range of hosts including Diptera, Lepidoptera, Coleoptera, Hemiptera, Isopoda, annelid worms and nematodes (Kelly, 1985). *Chilo* IV (IV6) has been designated as the type species of this genus, in view of the information available on its structure, replication and molecular biology (Francki *et al.*, 1991). In addition, there are two genera of iridoviruses isolated from vertebrates, the *Ranavirus* genus, of which the type species is frog virus 3 (FV3), and the *Lymphocystivirus* genus, for which the type species is flounder lymphocystis disease virus (FLCDV). FV3 and FLCDV share no nucleic acid homology, and their relationships with other members of the Iridoviridae are unclear (Willis, 1990). The genomes of FV3 and FLCDV are highly methylated in contrast to the structure of the invertebrate IV genomes that have been studied (Darai *et al.*, 1983, 1985; Essani, 1990).

In addition to the icosahedral virus particle structure, there are several other attributes which appear to unite

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members of the Iridoviridae. Foremost among these is the circular permutation and terminal redundancy of the genome. This is a feature unique among eukaryotic viruses, and suggests a 'headful' system of DNA packaging similar to that seen in bacteriophages. The common occurrence of a major structural protein (MSP) of around 50K in all of the iridoviruses examined to date is also notable, and has been suggested as a useful marker by which to standardize the orientation of iridovirus genomic maps, in much the same way as the polyhedrin or granulin genes are used for baculoviruses (Ward & Kalmakoff, 1991).

Vertebrate iridoviruses do not iridesce, but iridescence has habitually been the criterion for diagnosing invertebrate IV infection. The iridescent coloration is due to the crystalline arrangement of virus particles in heavily infected host tissues. However, iridescence can be a particularly poor criterion for diagnosing IV infections in certain invertebrate species (Stoltz *et al.*, 1968; Devauchelle, 1977). In a recent field study, two types of IV infection were detected in blackfly larvae (*Simulium* spp.), an obvious iridescent lethal form which was extremely rare and a covert, non-lethal form which was several orders of magnitude more common (Williams, 1993). A remarkable degree of variability in the restriction endonuclease (REN) profiles of iridovirus isolates from *Simulium* (Diptera: Simuliidae) larvae from the same river in the U.K. has been reported (Williams & Cory, 1993). With no two isolates appearing identical, the problem of determining the similarities between IVs from different host species adds to the complexity of defining taxonomic relationships.

The need for comparative studies on IVs is well recognized (Kelly *et al.*, 1978; Kelly, 1985; Willis, 1990; Ward & Kalmakoff, 1991; Stohwasser *et al.*, 1993; Schnitzler & Darai, 1993). This paper presents data on the relationships of 16 IVs distributed across all four genera, *Iridovirus*, *Chloriridovirus*, *Ranavirus* and *Lymphocystivirus*, in terms of REN profiles, dot-blot hybridization studies and comparisons of the major structural protein (MSP) gene. For reasons of clarity, we have followed the host/number system used by Kelly (1985) until the Discussion, wherein we propose a revised nomenclature for invertebrate IVs.

Methods

Collection, production and purification of iridoviruses. Of the many iridoviruses reported from invertebrate hosts, few have been kept for study. Consequently, a total of 16 iridoviruses including isolates from five insect orders, a crustacean order and two vertebrate isolates were gathered to give a representative sample of the available isolates for comparative studies (Table 1). Where possible, all IVs were grown in the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae), which is highly permissive to most invertebrate IVs and has habitually been used to prepare large quantities of IVs (Ward & Kalmakoff, 1991). Third instar *G. mellonella* larvae were injected with 5 µl of semi-pure virus suspended in an antibiotic solution (penicillin at 10000 IU/ml and streptomycin at 10 mg/ml). Larvae were maintained on artificial diet at room temperature until an obvious iridescence developed (some 10 to 20 days post-inoculation). Larvae were then sacrificed, and homogenized in sterile deionized water. Debris was pelleted by centrifugation at 1500 *g*, and the supernatant centrifuged at 10000 *g* for 15 min to produce a brilliant iridescent pellet of nearly pure virus. The pellet was resuspended in sterile water, a portion was taken for DNA extraction, and the remainder was stored at −20 °C.

Extraction, verification and REN characterization of DNA. DNA was extracted from each virus preparation using phenol–chloroform, followed by dialysis (Sambrook *et al.*, 1989). The concentration of the resulting DNA was measured by absorbance at 260 nm. DNA samples were stored at −20 °C. The DNAs from the original material and the *G. mellonella*-grown material were compared by digestion with *Hin*dIII (Boehringer Mannheim) following the recommended protocols, to check for changes during passage. The products were subject to electrophoresis using a 0·6% agarose gel in Tris–borate–EDTA buffer (Sambrook *et al.*, 1989) containing 0·3 mg/l ethidium bromide. DNA fragments were visualized under u.v. light at 312 nm. For REN characterization, DNA from each of the IVs (Table 1) was digested with *Hin*dIII, *Eco*RI and *Sal*I (Boehringer Mannheim) and fragments were resolved by 0·6% agarose gel electrophoresis. Using the program MolMatch (version 5.2; UVP Ltd, Cambridge, U.K.) the overall genome sizes were estimated. MolMatch also generated a coefficient of similarity (Dice coefficient) for pairwise comparisons of restriction fragment profiles according to the formula

$$S_D = 2m \times 100\,(\%)/a+b,$$

where m is the number of restriction fragments in common, (%) denotes the permissible fragment size variation across the gel and a and b are the total number of bands compared for each isolate (Grothues & Tümmler, 1991). The permissible variation was set at 2·5% for the *Hin*dIII gel. Similarity coefficients of less than two-thirds were not considered.

Southern blotting to locate the MSP gene. After photography, the *Hin*dIII gel was denatured, neutralized, blotted onto Hybond membrane (Amersham) as described by Sambrook *et al.* (1989), and baked at 80 °C for 2 h prior to Southern blot analysis. For blotting, a 1·4 kb *Sal*I fragment spanning the IV22 (*Simulium* IV) MSP gene was selected to be the probe. This fragment extends from bases 778 to 2178 in the sequence given by Cameron (1990) and when cloned into the plasmid pUC19 comprises 91% of the complete gene sequence. Approximately 200 ng of probe DNA together with 100 ng of phage lambda DNA were nick-translated with [^{32}P]dATP to a high sp. act. (Rigby *et al.*, 1979). The blot was prehybridized at 37 °C for a minimum of 4 h in 50% formamide and 50% hybridization buffer (50 mM-HEPES, 0·02% Ficoll 400, 0·02% BSA, 0·02% polyvinylpyrrolidone and 0·1% SDS) containing 100 µg/ml denatured salmon sperm DNA. Radiolabelled probe with an activity of approximately 10^7 to 10^8 c.p.m. was denatured by heat and added to a fresh mixture of 50% formamide and 50% hybridization buffer. Hybridization was performed overnight at 37 °C. The blot was washed twice at room temperature with 2 × SSC, followed by two washes, each of 1 h, in 2 × SSC at 65 °C, then autoradiographed overnight. Following this, the probe was stripped from the blot using 0·4 M-NaOH at 45 °C for 30 min, followed by two short washes in 200 mM-Tris–HCl pH 7·0, 0·1 × SSC, 0·1% SDS at room temperature. The blot was prehybridized as above, and then allowed to hybridize with the MSP probe but at a low stringency (20% formamide at 37 °C overnight, followed by washes in 2 × SSC at 40 °C) and autoradiography was done as before.

Table 1. *Iridovirus isolates used, including proposed names*

IV type	Original host species (Order)	Virus previously grown in	For this study virus grown in	Source of material	Characterization reference	Proposed names based on geographical origin
IV1	*Tipula paludosa* (Diptera)	*T. paludosa*	*G. mellonella*	IVEM (C. F. Rivers)	Tajbakhsh *et al.* (1986)	Plowden
IV2	*Sericesthis pruinosa* (Coleoptera)	*G. mellonella*	*G. mellonella*	IVEM	Day & Mercer (1964)	Tia
IV3	*Aedes taeniorhynchus* (Diptera)	*A. taeniorhynchus*	Used as supplied	S. Avery, USDA, U.S.A.	Wagner & Paschke (1977)	Vero Beach
IV6	*Chilo suppressalis* (Lepidoptera)	*Choristoneura fumiferana* cells (CF-124)	Supplied as DNA	G. Darai, Heidelberg, Germany	Delius *et al.* (1984)	Dazaifu
IV9	*Wiseana cervinata* (Lepidoptera)	Original material?	*G. mellonella*	IVEM	Ward & Kalmakoff (1987)	Nelson
IV10	*Witlesia sabulosella* (Lepidoptera)	Original material?	*G. mellonella*	IVEM	Fowler & Robertson (1972)	Nelson
IV18	*Opogonia* sp. (Coleoptera)	Original material?	Used as supplied	IVEM	Kelly & Avery (1974)	Nelson
IV21	*Helicoverpa armigera* (Lepidoptera)	*G. mellonella*	*G. mellonella*	IVEM	Carey *et al.* (1978)	Ntondwe
IV22	*Simulium* sp. (Diptera)	*G. mellonella*	*G. mellonella*	IVEM	Hibbin & Kelly (1980)	Aberystwyth
IV24	*Apis cerana* (Hymenoptera)	*A. mellifera*	Used as supplied	M. Allen Rothamstead, U.K.	Bailey *et al.* (1976)	Srinagar
IV28	*Lethocerus columbiae* (Hemiptera)	*G. mellonella*	*G. mellonella*	IVEM	Carey *et al.* (1978)	Ntondwe
IV29	*Tenebrio molitor* (Coleoptera)	*G. mellonella*	*G. mellonella*	IVEM	Black *et al.* (1981)	Fort Collins
IV30	*Helicoverpa zea* (Lepidoptera)	*H. zea*	*G. mellonella*	P. Christian, CSIRO, Australia	Stadelbacher *et al.* (1978)	Stoneville
IV31	*Armadillidium vulgare* (Isopoda)	*A. vulgare*	*G. mellonella*	B. Federici, Riverside, U.S.A.	Cole & Morris (1980)	Riverside
FV3	*Rana pipiens* (Anura)	Chick embryo	Used as supplied	A. M. Aubertin, ULP-INSERM, France	Lee & Willis (1983)	NA*
FLCDV	*Pleuronectes flesus* (Acanthopterygii)	Flounder lesions, field material	Used as supplied	B. Hill, MAFF, U.K.	Darai *et al.* (1983)	NA

* NA, Not applicable.

PCR products of the MSP gene. The ability of short oligonucleotide sequences to hybridize to each of the IVs was determined by PCR amplification. IV18 and IV28, being virtually identical to IV9 and IV21 respectively, were omitted from this study. The primers used were constructed using the IV22 (*Simulium* IV) MSP gene sequence (Cameron, 1990). The forward primer was designed to hybridize at bases 733 to 753 (5′ GGCGGCCCAACAGCAACAGC 3′) and the reverse primer (5′ GGCACAACCCATTCTACGACG 3′) to the complement of bases 1452 to 1431. The PCR reaction mixture comprised 42 µl water, 5 µl 10 × *Taq* buffer (NBL), 120 ng of each primer, 1 µl of 10 mM-dNTP, 1·5 units of *Taq* polymerase (NBL), and 5 ng of target IV DNA, to give a total volume of 50 µl. This mixture was overlaid with 50 µl of liquid paraffin. Water and salmon sperm DNA were used as negative controls. The annealing temperatures calculated for the forward and reverse primers were 64 °C and 62 °C respectively. The amplification protocol involved 40 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 30 s. Under these conditions the primers should theoretically anneal at high specificity, with a single G–C mismatch, or two A–T mismatches permitted in the forward primer, and a single A–T mismatch in the reverse primer. All PCR products were subject to digestion by *Xho*I (Boehringer Mannheim) following the manufacturer's instructions. A *Xho*I site is present at positions 1205 to 1210 of the IV22 MSP gene, and is also present at bases 1160 to 1165 of the IV1 (*Tipula* IV) MSP gene (Tajbakhsh *et al.*, 1990). PCR products before and after treatment with *Xho*I were visualized and their sizes measured by electrophoresis on a 1·2% agarose gel.

Dot-blot hybridization studies. Each DNA sample was heat-denatured, snap-chilled, and ammonium acetate was added to give estimated concentrations of 10 µg DNA/ml in 1 M-ammonium acetate. Hybond membrane was soaked in 1 M-ammonium acetate, and placed in a dot-blot apparatus (Bio-Rad) attached to a vacuum pump. Samples of each type of DNA (100 ng in 10 µl) were applied to four sequential wells of the apparatus. Denatured salmon sperm DNA (100 ng/well) was used as a control. The filter was then baked at 80 °C for 2 h. A total of 16 replicate filters were produced in this manner. Each of the 16 virus DNAs was individually radiolabelled by nick translation. Dot-blot filters were prehybridized and hybridized as described for Southern blots, but at a lower stringency of 40% formamide at 37 °C. Washes were performed in 2 × SSC at 55 °C prior to autoradiography. The autoradiograph was used to recover the hybridized DNA on the filters. Dots were cut from the filter and placed individually in scintillation tubes with 2·5 ml of scintillation cocktail (Ready Value; Beckman). Each tube was then counted for 3 min in a scintillation counter. From these results the mean percentage hybridization recorded for each DNA, relative to the homologous DNA, was calculated after correction for non-specific background hybridization to salmon sperm DNA.

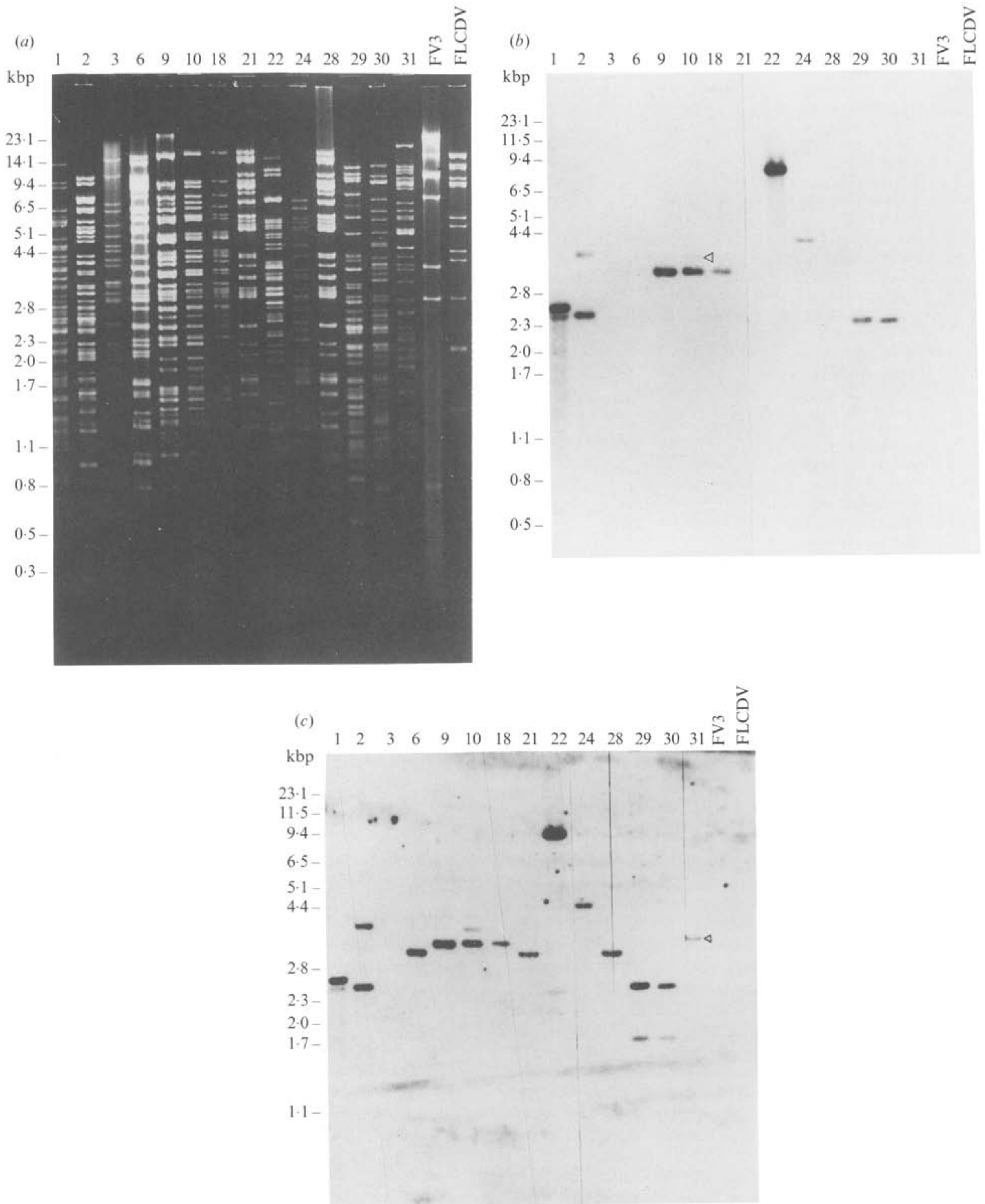

Fig. 1. REN profiles of IV DNA in 0·6% agarose gel following digestion by *Hin*dIII (*a*); Southern blot of *Hin*dIII gel probed at high stringency (50% formamide, 37 °C) with IV22 MSP gene fragment (*b*); or at low stringency (20% formamide, 37 °C) (*c*). Weak bands are highlighted by arrowheads.

Table 2. *Genomic sizes for iridovirus DNAs and sizes of* HindIII *fragments hybridized to the IV22 MSP gene probe*

IV type	Genome sizes estimated from gels (kbp)				Published value	Reference	*Hin*dIII fragments positive for MSP gene probe (kb)	
	*Hin*dIII	*Eco*RI	*Sal*I	average			High stringency	Low stringency
IV1	173·2	229·0	202·1	201·4	176	Tajbakhsh *et al.* (1990)	2·67, 2·56	
IV2	192·5	188·6	182·5	187·8	230	Kelly & Avery (1974)	4·02*, 2·53	
IV3	110·5	–	160·1	135·3	383	Wagner & Paschke (1977)	None	
IV6	214·2	207·8	–	211·0	209	Fischer *et al.* (1990)		3·1*
IV9	194·2	202·3	207·8	201·4	192·5	Ward & Kalmakoff (1987)	3·31	
IV10	176·5	194·7	191·9	187·7	–	–	3·74*, 3·32	
IV18	167·8	179·8	152·2	166·6	179	Kelly & Avery (1974)	3·30	
IV21	211·6	181·3	–	196·5	–	–		3·21
IV22	190·1	205·9	214·6	203·5	196	Hibbin & Kelly (1980)	7·18*	
IV24	130·2	150·0	–	140·1	–	–	4·31	
IV28	208·2	192·2	–	200·2	–	–		3·22
IV29	157·3	171·4	–	164·4	–	–	2·62†	1·73*
IV30	164·3	187·9	–	176·1	–	–	2·61*	1·73
IV31	201·9	195·9	196·6	198·0	184·8	Cole & Morris (1980)		3·56
FV3	107·3	–	–	107·3	107	Tondre *et al.* (1988)	None	
FLCDV	93·6	100·7	–	97·2	98	Schnitzler *et al.* (1990)	None	

* Double fragment.
† Triple fragment.

Results

REN profiles

The results of REN analysis were typified by the *Hin*dIII gel (Fig. 1*a*). It was apparent that IV9 (*Wiseana* IV), IV10 (*Witlesia* IV) and IV18 (*Opogonia* IV) were virtually identical. Likewise, IV21 (*Helicoverpa armigera* IV) and IV28 (*Lethocerus* IV) were almost indistinguishable. IV1 (*Tipula* IV) and IV2 (*Sericesthis* IV) did not show many REN profile similarities, indicating that these are more distantly related than previously suggested (Glitz *et al.*, 1968). As expected, IV3 (mosquito IV) and the vertebrate viruses FV3 and FLCDV appeared distinct from all the other isolates.

Fragment sizes were estimated from 0·6% gels and the sizes generated by the MolMatch program were fairly consistent among gels. They also compared favourably with IV genome sizes published elsewhere (Table 2). Owing to the paucity of restriction sites it was not possible to obtain genome sizes from the *Sal*I gels in some cases nor from the *Eco*RI gels in the case of IV3 and FV3. The coefficients of similarity for the *Hin*dIII fragments were as follows: IV1–IV2, 72%; IV1–IV18, 66%; IV1–IV30, 67·4%; IV2–IV10, 66·7%; IV2–IV30, 67·4%; IV9–IV10, 83·5%; IV9–IV18, 71·7%; IV10–IV18, 88·2%; IV21–IV28, 94·3%; IV22–IV30, 68·1%; IV24–IV30, 69·1%. These figures reinforce the apparent similarities seen in all the restriction profiles.

The genomic sizes observed were generally typical of IVs: 150 to 210 kbp for invertebrate IVs and around 100 kbp for the vertebrate IVs. Wagner & Paschke (1977) reported the IV3 (mosquito IV) genome size to be 383 kbp and a turquoise laboratory mutant strain to be 452 kbp. If correct, these would represent the largest recorded genomes of any DNA virus. The restriction profiles presented here suggest a significantly lower value for the normal mosquito IV (110 to 160 kbp). The hymenopteran IV (IV24) had a smaller than average genome size (130 to 150 kbp), but this is in line with other observations of IV genomic sizes, such as 144 kbp for *Scapteriscus aclectus* IV (Boucias *et al.*, 1987), or 153 kbp for *Simulium vittatum* IV (Erlandson & Mason, 1990).

Southern blot analysis

The presence of fragments that hybridized to the IV22 MSP gene probe was demonstrated for all the viruses tested with the exception of IV3 and the vertebrate viruses FV3 and FLCDV. At high stringency, between one and three bands of IV1, IV2, IV9, IV10, IV18 and IV22 hybridized strongly to this probe (Fig. 1*b*). IV9, IV10 and IV18 were virtually indistinguishable in this analysis. IV24, IV29 and IV30 hybridized consistently but more weakly to the probe. Both IV29 (*Tenebrio* IV) and IV30 (*Helicoverpa zea* IV) showed identically sized fragments carrying the MSP gene. The virtually identical sizes of the *Hin*dIII fragments (Fig. 1*c*) from IV6, IV21 and IV28, which consistently hybridized with the MSP

gene probe (at low stringency only), suggest they are related (Table 2). The IV3, FV3 and FLCDV consistently failed to hybridize to the probe at either stringency.

PCR products from the MSP gene region

The ability of the short oligonucleotide PCR primers to recognize homologous sequences was evident for IV1, IV2, IV9, IV10, IV22 and IV29 (Fig. 2). The size of PCR products was as predicted (approximately 719 bp) and the variation in size among the products of different IVs was too small to quantify accurately. Only the PCR products of IV1 and IV22 were cleaved by *Xho*I, giving two fragments which migrated in accordance with the 472 bp and 247 bp fragments predicted from the published restriction site. The PCR product of IV10 appeared only partially digested despite long incubation times using an excess of enzyme.

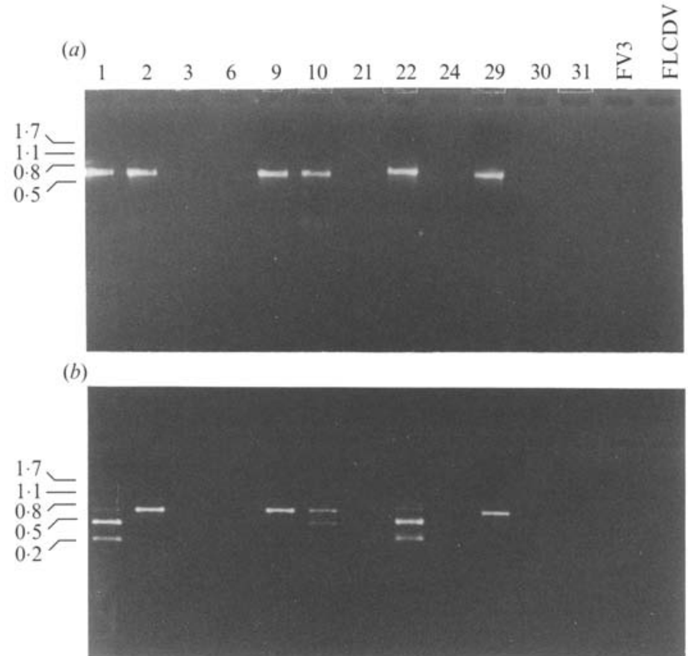

Fig. 2. PCR products from amplification of a 719 bp fragment of the MSP gene region (*a*) using primers derived from the IV1 and IV22 MSP gene sequences (Tajbakhsh *et al.*, 1990; Cameron, 1990). (*b*) Each product was also tested for the presence of a *Xho*I restriction site, predicted to yield fragments of approximately 472 and 247 bp (IV18 and IV28 omitted).

Dot-blot hybridization

The tendency for hybridization studies to underestimate actual levels of sequence homology is well recognized. Nevertheless, at the intermediate stringency of the dot-blot hybridization, the IVs tested could be clearly divided into five groups (Table 3). The chloriridovirus IV3, the isopod virus IV31, and both the vertebrate viruses, FV3 and FLCDV, failed to hybridize to any probe except homologous DNA. IV21 and IV28 were shown by REN profiles to be virtually identical, and both hybridized with high affinity to IV6 (*Chilo* IV) but to no other isolates. This affinity was also observed in the reciprocal blots with radiolabelled IV6. Thus, IV6, IV21 and IV28 form a separate and distinct group. Finally, the nine remaining isolates, IV1, IV2, IV9, IV10, IV18, IV22, IV24, IV29 and IV30, all displayed various degrees of hybridization to each other.

Discussion

A consistent pattern has been observed in the results of the studies described here. The majority of the IVs fell into two interrelated groups, with mosquito and isopod

Table 3. *Dot-blot hybridization values (%) for vertebrate and invertebrate iridoviruses, relative to homologous DNA after correction for background*

	IV1	IV2	IV3	IV6	IV9	IV10	IV18	IV21	IV22	IV24	IV28	IV29	IV30	IV31	FV3	FLCDV
IV1	100															
IV2	41	100														
IV3	0	< 1	100													
IV6	0	< 1	0	100												
IV9	20	46	0	0	100											
IV10	17	41	0	0	91	100										
IV18	17	55	0	0	100	89	100									
IV21	1·2	< 1	0	42	0	0	< 1	100								
IV22	33	22	0	0	28	27	20	0	100							
IV24	13	24	0	0	22	17	18	< 1	10	100						
IV28	1	< 1	0	44	0	0	< 1	100	< 1	1	100					
IV29	13	26	< 1	1·1	32	31	28	0	15	27	0	100				
IV30	11	36	0	0	33	32	32	0	23	24	0	56	100			
IV31	< 1	1·4	0	2·4	0	0	0	1·6	0	0	1·4	0	< 1	100		
FV3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	
FLCDV	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100

IVs appearing distinct and separate in addition to the vertebrate viruses. This is in broad agreement with the serological studies summarized by Kelly *et al.* (1979) in which one main serogroup comprised IV1, IV2, IV9, IV10, IV16, IV18, IV21, IV22, IV23, IV25 and IV28. IV6 and IV24 were described as serologically distinct from other isolates and from each other. IV29 was serologically related only to IV1, IV2, IV22, IV23 and IV25. It is appropriate to consider the implications of our findings for each isolate in turn.

IV1 (*Tipula* IV) was, as the name implies, the first IV discovered. The restriction fragment profiles and location of the MSP gene are in agreement with those given by Tajbakhsh *et al.* (1990). The successful amplification and *Xho*I restriction of the PCR product was expected since the published sequences of the IV1 and IV22 (*Simulium* IV) MSP genes are virtually identical. IV1 belongs to the largest group of inter-related isolates. IV2 (*Sericesthis* IV) has often been used in comparative studies with IVs that have been discovered subsequently (Bellet & Fenner, 1968; Glitz *et al.*, 1968; Kalmakoff *et al.*, 1972; Kelly & Avery, 1974; Elliott *et al.*, 1977; Kelly *et al.*, 1979; Moore & Kelly, 1980). IV1 and IV2 have been described as strains of the same virus (Glitz *et al.*, 1968; Willis, 1990). Although a relationship between these viruses is apparent, given the hybridization and PCR product results presented here to consider them as strains of the same virus is an overstatement. No similarities between IV3 (mosquito IV) and any of the other IVs have been observed. This representative of the *Chloriridovirus* genus remains discrete, and separate from the other invertebrate and vertebrate IVs.

In agreement with the serological studies summarized in Kelly *et al.* (1979), *Chilo* IV (IV6) appeared distantly related to most of the other members of the *Iridovirus* genus with the exception of IV21 and IV28. Recently, Stohwasser *et al.* (1993) have used oligonucleotide primers representing conserved regions of the IV1 and IV22 MSP gene sequences (Tajbakhsh *et al.*, 1990; Cameron, 1990) for PCR amplification and identification of the MSP gene of IV6. The gene showed a DNA sequence similarity of 66·1% when compared to the gene from IV1. A noticeable difference in the characterization of IV6 compared to the serological summary of Kelly *et al.* (1979) was detected in its relationship to IV21 and IV28. IV21 (*Helicoverpa armigera* IV) and IV28 (*Lethocerus columbiae* IV) have been acknowledged to be virtually indistinguishable from one another serologically since they were first described (Carey *et al.*, 1978), and were assigned to the main serogroup of related viruses which included IV1, IV2 and IV22, but not IV6. However the results of this study indicated a close similarity between IV6, IV21 and IV28. This was apparent in the dot-blot hybridization results and their common affinity for the IV22 MSP gene probe, which hybridized to similarly sized fragments at low stringency. To elucidate this problem, an IV6 sample with a known history was obtained from Dr P. Christian (CSIRO, Canberra, Australia). The *Eco*RI restriction profile of this IV was compared to that of IV21. Virtually no differences were observed (data not shown). This suggests that either IV21 and IV28 from the Institute of Virology and Environmental Microbiology (IVEM) virus collection have both been contaminated by a strain of IV6, or that these strains are indeed closely related to IV6. The sample used in this study probably represents the most faithful source of IV28, coming from a bottle in the IVEM collection marked '*Lethocerus columbiae* Iridescent Virus (IV28) Single passage *Galleria* – 1 rate gradient – Pure'. It has not been possible to trace any alternative source of IV21 or IV28 for comparison, so the exact identity of IV21 and IV28 will only be verified if the original material exists in storage, or if the viruses are re-isolated, Nevertheless, it is clear that IV6 is not closely related, either serologically or genetically, to the other insect IVs with the possible exceptions of IVs 21 and 28.

IV9, IV10 and IV18 appear to be strains of the same virus. All three isolates were discovered at the same locality on New Zealand's South Island, but in different hosts. IV9 has been compared with another New Zealand IV, IV16 from *Costelytra zealandica* (Coleoptera: Scarabeidae: Kalmakoff *et al.*, 1990). Regions of non-homology between these two viruses have been described, accounting for 12·5% of the IV9 genome and 40% of the IV16 genome. Between these regions there are large sections containing repeat sequences, as reported for other IVs (Goorha & Murti, 1982; Darai *et al.*, 1985; Tajbakhsh *et al.*, 1986; Schnitzler *et al.*, 1987; Fischer *et al.*, 1990), although the precise nature of these sequences has not been described for IV9. The PCR product of IV10 was only partially cleaved by *Xho*I. This suggests that there was a mixture of PCR products from this isolate, some containing and some lacking the *Xho*I site, which may have resulted from the non-clonal nature of the original field-collected material used here.

As a result of personal interest, and the availability of sequence data, much of this study has been made with reference to IV22 (*Simulium* IV, from the U.K.). This was not a poor choice of candidate for comparative studies as it belongs to the largest and most interrelated group of IVs. It has recently been shown that IVs isolated from sympatric *Simulium* larvae varied distinctly in terms of restriction profiles (Williams, 1993; Williams & Cory, 1993). Whether this degree of variation is commonly seen in IV populations from other invertebrate species is not known, although a comparable situation seems to exist among the IV9, IV10 and IV18 isolates from different insect orders. Another isolate

Genus *Iridovirus*			Genus *Chloriridovirus*
Polyiridovirus complex Type: Plowden IV	Oligoiridovirus complex Type: Dazaifu IV	Crustaceoiridovirus complex Type: Riverside IV	Type: Vero Beach IV
Aberystwyth (IV22, IV25)	Dazaifu (IV6)	Riverside (IV31, IV32)	Vero Beach (IV3)
Fort Collins (IV29)	Ntondwe (IV21, IV28)		
Nelson (IV9, IV10, IV18)			
Plowden (IV1)			
Srinagar (IV24)			
Stoneville (IV30)			
Tia (IV2)			
Timaru (IV16, IV19)			
Uitenhage (IV23)			

Fig. 3. Revised system of nomenclature and classification of invertebrate IVs. These are named according to place of origin and allocated to discrete complexes of interrelated isolates.

from a *Tipula* sp. (IV25) has been described as virtually indistinguishable from IV22, serologically or in terms of polypeptide profile (Elliott *et al.*, 1977).

According to the serological grouping of Kelly *et al.* (1979), IV24 (*Apis* IV) is unrelated to any of the other invertebrate IVs. However genomic analyses indicated that this was not the case. The moderate affinity for the IV22 MSP probe in the Southern blot analysis and the appreciable levels of DNA hybridization measured place this isolate within the main group of interrelated IVs. Likewise, IV29 (*Tenebrio* IV), previously described as having serological similarities only with IV1 and IV22 (and two other isolates not included here), can now be integrated into the main interrelated group. IV30 DNA hybridized with all members of the main IV group, particularly to IV29, and also showed similarities to IV29 in terms of the location and strength of hybridization to the IV22 MSP gene probe.

The isopod IV (*Armadillidium* IV), IV31, was the only IV examined with a non-insect invertebrate host. The distinct nature of this virus was apparent in all the studies with the exception of the weak hybridization of a fragment to the IV22 MSP gene probe in Southern blot analysis. In an early description of an IV from *Armadillidium vulgare* and *Porcellio scaber*, Cole & Morris (1980) reported only a distant serological relationship between isopod IV, IV1 (*Tipula* IV) and an IV from *Phylophaga anxia* (Coleoptera; Poprawski & Yule, 1990). Since the review of Kelly (1985) in which two species of terrestrial isopod are given as IV hosts (*A. vulgare*, IV31; *Porcellio dilatatus*, IV32), a further seven species of North American woodlouse have been observed with patent IV infections (Cole & Morris, 1980; Federici, 1984; Grosholz, 1992). The majority of these have not yet been characterized. Recently, marine crustacea have also been suspected as being hosts to IV infection (Montanie *et al.*, 1993; Lightner & Redman, 1993), although no comparative work has yet been described.

There was no evidence in this study to alter the conventional view that the vertebrate IVs, FV3 and FLCDV, are only distantly related to any of the invertebrate IVs and to each other. These isolates were consistently negative in all the tests performed, even the low stringency Southern blot. However, FLCDV and IV6 show very similar overall levels of DNA sequence identity to the IV22 MSP gene, of approximately 69·3% and 73·4% respectively. The reason why IV6 hybridized to the MSP gene probe, whereas FLCDV did not, lies in the length of the continuous base homology. In a comparative study, Schnitzler & Darai (1993) showed that regions of high homology are located close together in the IV6 MSP gene but scattered over the entire gene of FLCDV. They also reported MSP amino acid sequence identity/similarities of 33·8/50·3% for IV1, 34·2/49·1% for IV22 and 29·5/53% for IV6 when compared to the deduced homologous FLCDV sequence.

Given the accumulation of information on IVs and the confusion that may arise over nomenclatures based on history and host species, the current system of IV classification should now be revised. Invertebrate IVs are classified on two levels: the number/host system of Tinsley & Kelly (1970) and the generic division according to size. Sadly, for the vast majority of isolates, material

has been neither kept nor characterized. Most reports of IV infections in natural populations have described pathology, particle morphology, and occasionally some physico-chemical properties of the virus. Only 23 isolates have been characterized to a level which includes REN profile and genome size, comparative serological studies, or detailed physico-chemical and morphological description. Of these 23 isolates, seven appear to be strains of previously described virus species, albeit from different hosts. This highlights the problems associated with naming isolates after the hosts they were derived from. Such procedures currently cause great confusion in other virus families, for example the Baculoviridae. The type numbering system is equally unhelpful. Consequently we propose a new system, which has precedents in other families including the Reoviridae, Bunyaviridae, Arenaviridae and Rhabdoviridae, where invertebrate IVs are named according to their reported place of discovery. Thus, *Tipula paludosa* IV (IV1), discovered at Plowden in the U.K., becomes Plowden iridovirus. The strains of *Wiseana cervinata* IV (IV9, IV10 and IV18), discovered near Nelson in New Zealand, become Nelson iridovirus, and so on. The proposed names given in Table 1 are based on the closest main settlement to the point of discovery, or the location of the reporting institution where the geographical origin is unknown (for example Stoneville in the case of IV30).

From the results of this and previous studies, it would be appropriate to subdivide the *Iridovirus* genus into three distinct complexes (Fig. 3). The first complex comprises the major interrelated group of IV1, IV2, IV9, IV10, IV18, IV22, IV24, IV29 and IV30. Owing to their prevalence we propose to call this hybridization group the Polyiridovirus complex, with the type isolate being Plowden IV (IV1, *Tipula* IV). The second complex would include Dazaifu IV (IV6, *Chilo* IV) being the type species of the *Iridovirus* genus and tentatively include Ntondwe IV (IV21 and IV28). Because it has fewer members, we propose to call this the Oligoiridovirus complex. The third group, composed of the crustacean IV represented by Riverside IV (IV31, *Armadillidium* IV) is named the Crustaceoiridovirus complex.

This system has a number of additional advantages. New IV isolates could be easily assigned to one of the complexes, by comparative hybridization (whole DNA or oligonucleotide PCR primers) to Plowden (IV1) or Dazaifu (IV6), the two most readily obtainable and best characterized of the invertebrate IVs. Crustacean isolates could be compared in a similar manner to Riverside IV (IV31). Once allocated to a particular complex, a number of assumptions can be made, for example with respect to the MSP gene, which can then be tested using the sequences published for members of the Polyiridovirus (Tajbakhsh *et al.*, 1990; Cameron, 1990) and Oligoiridovirus (Stohwasser *et al.*, 1993) complexes. Comparative studies could then be undertaken involving other members of the complex.

Future descriptions of novel isolates may encounter two problems. First, restriction fragment length polymorphisms will probably be observed and second, it is feasible to compare only a discrete range of isolates in order to categorize the novel isolate in question. We therefore propose the following criteria for characterization. The Plowden and Dazaifu IV type isolates should be used, with Riverside IV as well if the novel isolate is from crustacea.

To unite isolates under a common name the criteria are (i) Dice coefficients exceeding 80% from a number of REN profiles, (ii) DNA hybridization values exceeding 80% under the conditions given above and (iii) common restriction fragments bearing the MSP gene.

To allocate an isolate to a hybridization complex the criteria are (i) DNA hybridization values consistently exceeding 5% with members of the complex under the conditions given above and (ii) DNA hybridization values consistently less than 5% with isolates from other complexes.

Including published serological results concerning additional IVs, it is appropriate to place Timaru IV (IV16 from *Costelytra zealandica* and IV19 from *Odontria striata*; Ward & Kalmakoff, 1991; Kalmakoff *et al.*, 1990; N. McMillan, personal communication) and Uitenhage IV (IV23 from *Heteronychus arator*; Kelly *et al.*, 1978) within the Polyiridovirus complex to create a revised system of invertebrate IV classification shown in Fig. 3. There is also strong evidence that IV22 and IV25 can be grouped under a common name, Aberystwyth IV (Elliott *et al.*, 1977). Both of the vertebrate genera (*Ranavirus* and *Lymphocystivirus*) remain unchanged by the results of this study.

An unfortunate situation exists among isolates of the genus *Chloriridovirus*. Although the most numerous reports of IVs come from mosquitoes and related dipteran species, only one isolate (IV3, Vero Beach IV) has been characterized. There has also been a tendency to place any IV isolate reported from mosquitoes and midges into the *Chloriridovirus* genus, irrespective of particle size and in the absence of any comparative studies (Matthews, 1982; Francki *et al.*, 1991). The reasons for such unsystematic allocations are not clear.

We believe that the system of nomenclature and classification by complex described here will clarify a confused taxonomy within the Iridoviridae and should greatly simplify the future characterization of IV isolates.

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