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SHORT COMMUNICATION

DNA restriction fragment polymorphism in iridovirus isolates from individual blackflies (Diptera: Simuliidae)

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Iridescent viruses (IVs) are icosahedral particles containing double-stranded DNA which assemble in host cytoplasm. Over 30 IVs have been isolated from invertebrate (mainly insect) hosts world-wide. They are classified in two genera, based on size. Members of the genus *Chloridovirus* (180–200 nm diameter) have been isolated only from Diptera (mainly mosquitoes) whereas the genus *Iridovirus* (*c.* 130 nm diameter) has a broader host range in several insect Orders, Crustacea, nematodes and an annelid worm (Kelly, 1985). Heavily infected hosts typically display a blue-green or lilac iridescence due to the formation of crystalline arrays of virus particles within host tissues. Light striking these viral arrays is subject to interference in the form of Bragg reflections resulting in the opalescent hue. This simplifies the diagnosis of patent infections because the larvae become bright blue. Historically, this has also been the criterion for determining whether or not an insect harbours an iridovirus infection and, as such, the incidence of IV infections in invertebrate populations has usually been regarded as low. The majority of reports of IV infection describe a single patently infected indvidual in a local population of several hundreds or thousands of apparently healthy conspecifics. For most IVs, even the route of infection remains unknown. The low frequency with which patent infections develop following ingestion of large doses of virus contrasts strongly with the high infectivity of virus particles when injected, and has led to suggestions of transmission via wounds or cannibalism, or carried into the host haemocoel by nematode parasites. As an interim measure, IVs are classified by host and number (e.g. *Tipula* IV = IV1, *Sericesthis* IV = IVs, etc.) according to the sequence of discovery, without regard for the *Iridovirus*/*Chloridovirus* division (Tinsley & Kelly, 1970).

Iridovirus type 22 was isolated from a *Simulium* blackfly larva (Diptera: Simuliidae) collected in the River Ystwyth, Wales (U.K.) in the early 1970s. Only 12 patently infected larvae were found during a 2-year study in which some 6–10 million larvae were examined (Batson *et al.*, 1976; Kelly *et al.*, 1978). Since that time laboratory studies have considered replication in cell culture (Kelly, 1976, 1980; Brown *et al.*, 1977), immunological relatedness and comparisons with other IV isolates (Kelly *et al.*, 1978, 1979; Kelly, 1980), characterization of DNA (Hibbin & Kelly, 1981) and the location and sequence of the gene coding for the major structural protein of the viral capsid (Cameron, 1990), but no data exist on the ecology or host relationships of this virus.

Insect viruses are typically characterized using DNA cleavage patterns produced following treatment with restriction endonucleases (REN). This communication describes variation in the REN profiles of iridovirus isolates from individual simuliid larvae taken from the River Ystwyth in Wales. The diversity of REN profiles obtained indicates marked variation in the iridovirus population exploiting individual blackfly hosts. This in turn has consequences for the ecological study of iridovirus infections in blackfly populations.

For this investigation, patently infected *Simulium* larvae attached to stones in fast-flowing sections of the River Ystwyth were collected on two occasions. On 18 September 1991, a single blue larva was taken from Pentre-llyn (Ordnance Survey map 135 ref. 619, 757) some 7 km from the river estuary (water temperature 14.7°C, flow velocity 1.1 m s^{-1}). No patently infected larvae were found in March or April but, on 26 May 1992, two blue larvae were collected at Pentre-llyn and five infecteds at Abermagwr (ref. 667 729) some 13 km from the estuary (water temperature 18°C, flow velocity 0.9 and 1.4 m s^{-1} respectively). Field-collected larvae were placed individually into Eppendorf tubes containing 0.2× dilution of standard antibiotic solution (10 mg/ml streptomycin and 10,000 iu/ml penicillin), returned to the laboratory and frozen until required.

Patently infected larvae were measured and examined with a binocular microscope before being individually homogenized in 120 µl of 1× antibiotic solution in a sterile Eppendorf tube. Larval debris was pelleted at 1500 *g* for 5 min in a microcentrifuge, leaving a milky viral suspension.

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To bulk-up each isolate, early third instar *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), a permissive host, were injected with 10 µl of the suspension into the haemocoel and maintained on artificial diet at room temperature. Uninfected blackfly larvae from rivers near Oxford were treated in the same manner and injected into *G.mellonella* to control for contamination. When the *G.mellonella* larvae developed an obvious opalescence, they were chilled until death and homogenized in 1–2 ml of sterile TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Debris was pelleted at 1500 *g* and the supernatant spun at 10,000 *g* for 15 min. This second pellet was distinguished into two parts: an upper brown 'skin' which was simply detached by agitation on a whirlimixer for a few seconds, and a brilliantly iridescent lower part of nearly pure virus. After discarding the skin and old supernatant, the viral pellet was resuspended in sterile TE buffer, a portion taken for DNA extraction and the remainder stored at −20°C.

Extraction of viral DNA from *Galleria*-grown virus was achieved by adding 400 µl of the virus suspension to 600 µl of sterile TE buffer, 10 µl of 10% SDS and 10 µl of Proteinase K (10 mg/ml). After incubation for 18 h at 37°C, remaining viral particles were pelleted and DNA extracted by successive treatment with TE-saturated phenol, phenol–chloroform and chloroform/isoamyl alcohol (1:25). DNA was then dialysed in three changes of TE buffer at 4°C overnight and aliquots were stored at −20°C. For REN digestion, the DNA produced from *Galleria*-grown virus was treated with Hind III following the recommended protocols and separated on 0.6% agarose by electrophoresis overnight at 30 V in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) containing 0.3 mg/l ethidium bromide. DNA fragments were visualized and photographed in UV light at 312 nm.

All the infected larvae appeared consistent with the morphological description given in Davies (1968) for *Simulium variegatum* Meigen. This was the dominant species of larva in the Ystwyth during May, followed by *S.reptans* (L.). In September, *S.intermedium* Roubaud, *S.ornatum* Meigen and *S.variegatum* were identified. The mean length (±SE) of infected larvae was 4.55 ± 0.32 mm, i.e. they conformed to the normal size of third or fourth instar conspecifics. None of the *G.mellonella* larvae injected with uninfected simuliids from rivers around Oxford showed any signs of iridescence; there was no cross-contamination.

Examination of the REN profiles (Fig. 1) shows that no two isolates were identical when cut with Hind III. The two isolates from Pentre-llyn collected in May (lanes 3 and 4) showed only minor differences from one another; profile similarity of each isolate to the standard IV22 (lanes 1 and 10) was evident for all but the specimen collected in September 1991 (lane 2). The most obviously conserved band was approximately 6.5 kbp in size and appeared in all profiles except lane 2. IV22 has been passaged numerous times through *G.mellonella* without detectable changes in the REN profile, indicating a degree of stability in passage through this host.

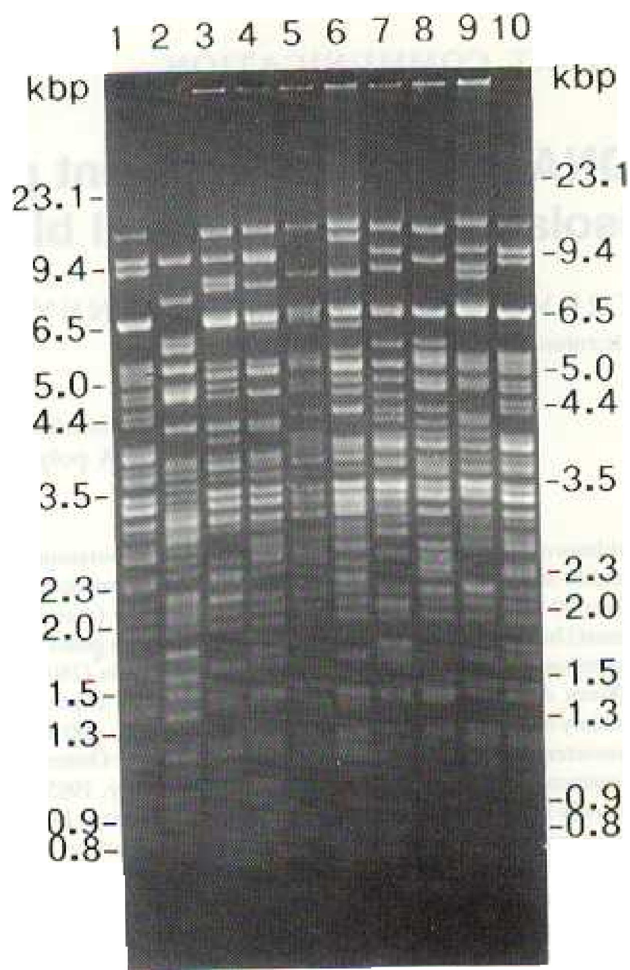

Fig. 1. Restriction endonuclease profiles of iridovirus isolates from individual *Simulium* larvae, grown in *G.mellonella*. Lanes 1 and 10 = Iridovirus type 22; lane 2 = Pentre-llyn September isolate; lanes 3 and 4 = Pentre-llyn May isolates; lanes 5–9 = Abermagwr May isolates. DNA was digested with Hind III and fragments separated by 0.6% agarose gel electrophoresis, stained with ethidium bromide and visualized in UV light. All sizes in kilobase pairs.

The high number of fragments produced by Hind III digestion, 42 in the case of IV22 (Hibbin & Kelly, 1980), makes this enzyme a relatively sensitive indicator of variation in isolate sequences when compared to a more infrequent cutter (e.g. Bam HI, Sal I, Pst I, etc.).

In other insect viruses, such as baculoviruses, the appearance of sub-molar bands in REN profiles of field isolates has been reported frequently and is taken as evidence of genotypically heterogeneous infection (Smith & Crook, 1988). Crawford *et al.* (1986) found that most of the changes in REN profiles of geographically distinct *Oryctes* (Rhinoceros beetle) baculovirus isolates were due to small insertions or deletions, and were mainly associated with regions containing reiterated sequences. Such changes could occur by insertion of host DNA, duplication of viral

sequences, or point mutations (Brown *et al.*, 1985). In addition, recombination between related, co-infecting viruses (Smith & Summers, 1980) or between the progeny of mixed infections (Summers *et al.*, 1980) has been detected *in vitro*, and may be common in field populations of viral pathogens.

The obvious question that arises from these findings concerns the adaptive significance of the observed degree of genomic variation. Is the variation localized within specific hyper-variable regions of the IV genome and what role do these regions play in determining viral transmission or replication strategies in simuliid populations? Will IVs from other invertebrates show a similar degree of variation? The repeated observation of sub-molar fragments in REN profiles of pooled iridovirus isolates (Tajbakhsh *et al.*, 1986; Ward & Kalmakoff, 1987; Erlandson & Mason, 1990) suggests this may be so. In addition, Ward & Kalmakoff (1991) noticed the elimination of sub-molar fragments following serial passage of *Wiseana cervinata* (Lepidoptera: Hepialidae) iridovirus IV9 through *G. mellonella*; presumably a mixed population of isolates was initially present in the field-collected material and, through serial passage, one particular strain showed competitive elimination of rival variants.

Our findings make accurate comparison of the relationships between IVs isolated from different taxa more complex than previously perceived, and highlight the importance of avoiding virus pooling from more than one infected individual in order to obtain pure isolates from each host species. When attempting to find non-patent iridovirus infections by detecting viral DNA in host tissues, either by dot-blot, *in-situ* hybridization or the polymerase chain reaction (PCR), it is essential that the probe be targetted at a highly conserved region of the genome, such as the major structural protein gene. Use of a probe for a more variable region will probably fail to detect a proportion of the infecteds and so underestimate the prevalence and misinterpret the impact of iridescent viruses in host populations.

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