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Expansion of family Reoviridae to include nine-segmented dsRNA viruses: Isolation and characterization of a new virus designated aedes pseudoscutellaris reovirus assigned to a proposed genus (Dinovernavirus)☆

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Abstract

Family Reoviridae is known, by definition, to contain dsRNA viruses with 10–12 genome segments. We report here the characterization of the first member of this family with a nine-segmented genome. This virus was isolated from *Aedes pseudoscutellaris* mosquito cells and designated aedes pseudoscutellaris reovirus (APRV). Virions are single-shelled with turrets but are non-occluded by contrast to cypoviruses. APRV replicates in various mosquito cell lines, but not in mice or mammalian cells. Complete sequence analysis showed that APRV is phylogenetically related to cypoviruses, fijiviruses and oryzaviruses. The maximum amino acid identities with cypoviruses, oryzaviruses or fijiviruses in the polymerase, are compatible with values observed between these genera and lower than values within a given genus. This suggests that APRV should be classified within a new genus that we designated Dinovernavirus (sigla from D: Double-stranded, i: insect, nove: nine from the latin "novem", rna: RNA, virus) in family Reoviridae.

Keywords: ds-RNA viruses; Reoviridae; Single-shelled turreted virus; Nine-segmented dsRNA genome; Aedes pseudoscutellaris reovirus; Dinovernavirus

Introduction

Since the original characterization of an orthoreovirus by Sabin in 1959 (Sabin, 1959), numerous other viruses have been identified with genomes composed of multiple (10, 11 or 12) segments of linear double-stranded RNA (dsRNA). These viruses have been isolated from a wide range of host species, including mammals, birds, reptiles, fish, crustaceans, marine protists, insects, ticks, arachnids, plants and fungi. They currently include a total of 75 virus species, which are classified as members of 12 different genera within the family Reoviridae, with a further ~30 tentative or 'unassigned' species reported to date (Joklik, 1983; Mertens et al., 2005; Brussaard et al., 2004). The genera *Coltivirus*, *Orbivirus* and *Seadornavirus*, include arboviruses that infect animals (Mertens et al., 2005; Attoui et al., 2005a, 2005b). Many of the plant reoviruses (*Fijivirus*, *Oryzavirus* and *Phytoreovirus*) are also transmitted between their hosts by vector insects (such as leafhoppers) and are therefore plant arboviruses. However, viruses belonging to the other genera (*Aquareovirus*, *Cypovirus*, *Idnoreovirus*, *Mycoreovirus*, *Orthoreovirus* and *Rotavirus*) are thought to be transmitted horizontally or vertically between individual host organisms, often by an oral/fecal route.

Reoviruses are usually regarded as non-enveloped, although some can acquire a transient membrane-envelope during virion

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morphogenesis or cell exit (Murphy et al., 1968; Martin et al., 1998; Mertens et al., 2005; Owens et al., 2004; Mohd Jaafar et al., 2005a, 2005b). Reovirus particles have icosahedral symmetry, with a diameter of approximately 60 to 85 nm and can be subdivided into two groups, the 'turreted', and 'non-turreted' viruses. Core particles of the non-turreted viruses have a protein-bilayer structure, with an inner 'subcore' layer that has pseudo $T = 2$ symmetry (also described as $T = 1$), surrounded by a core-surface layer with $T = 13$ symmetry. This gives the cores a smooth or bristly surface appearance and a relatively uniform diameter (e.g. rotaviruses or orbiviruses—Grimes et al., 1998; Baker et al., 1999; Mertens et al., 2005). In contrast, cores of the turreted viruses have 12 icosahedrally arranged projections (called turrets or spikes), situated on the core surface, one at each of the 5-fold axes (e.g. orthoreoviruses or cypoviruses) (Baker et al., 1999; Hill et al., 1999; Nibert and Schiff, 2001). In contrast to most other reoviruses, the cypoviruses lack an outer-capsid layer, having only a single-shelled, turreted structure.

Reoviruses have previously been isolated from tick cells derived from wild caught ticks. These include *St. Croix River virus* (SCRV, an orbivirus), which was isolated from IDE2 tick cells, established from eggs of wild caught *Ixodes scapulris* tick (Attoui et al., 2001). We report the isolation of a novel single-shelled dsRNA virus from a continuous mosquito cell line established in 1974 (AP61 cells from *Aedes pseudoscutellaris*—Varma et al., 1974). The virus is identified as aedes pseudoscutellaris reovirus (APRV). The virus, which persistently infects AP61 cells but shows no visible cytopathic effect. Electron microscopy of purified APRV-1 showed a well defined, turreted and single-shelled capsid. The only single-shelled turreted reoviruses that have previously been identified belong to the genus *Cypovirus*, although these normally have a 10-segmented dsRNA genome. However, unlike APRV-1, which is a non-occluded virus, the cypoviruses can usually become occluded within crystalline protein bodies called polyhedra. Electrophoretic analysis of the APRV-1 genome showed only nine genome segments, making it unique within the family Reoviridae.

Results

Nature of the poly-segmented genome

The DNase treatment of the genome of APRV did not affect its integrity as analyzed on agarose, showing that the genome is not made of DNA. When the genome was treated with RNase A at low ionic strength (0.1× SSC), this resulted in the digestion of the genome, showing that it is made of RNA. At high ionic strength (2× SSC: condition which does not allow strand separation), there was no alteration of the agarose migration pattern. These results showed that the genome of APRV is constituted of dsRNA.

The RNA extract from infected cells run on a 1% agarose gel in TAE buffer showed three groups of dsRNA segments: long segments migrating as three distinct bands, intermediate segment running as a single band and short segments running as a single band. These corresponded to the genome of APRV.

The PAGE profile of the genome shown in Fig. 1 revealed 9 distinct dsRNA bands. The densitometry analysis of the separated bands revealed the presence of equimolar amounts of each band. This was deduced after correcting the peak intensity of each band to its real size (determined by sequencing). These data suggested that each genome contained a single copy of each segment.

Therefore, the PAGE profile of the genome shown in Fig. 1 revealed 9 distinct dsRNA segments, 5 belonging to the long class designated Seg-1 to Seg-5, one belonging to the intermediate class designated Seg-6 and 3 belonging to the short class and designated Seg-7 to Seg-9.

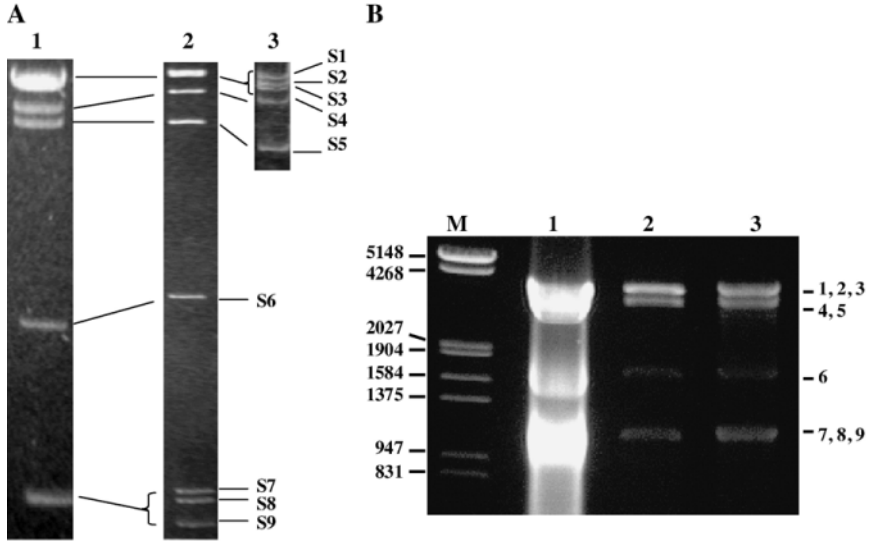

Fig. 1. Agarose and PAGE electrophoretic profiles of APRV genome. (A) APRV genome (extracted from infected AA23 cells) electrophoretic profile in: (1) 1.2% agarose in TAE buffer, (2) in 10% polyacrylamide gel in tris–glycine buffer (run for 24 h at 20 mA) and (3) in 10% polyacrylamide gel in tris–glycine buffer (run for 72 h at 20 mA) to separate into 3 distinct band the top group of dsRNA. (B) The genome of APRV in AA23, C6/36 and A20 cells. Lane M: size markers labeled in bp; Lane 1: dsRNA of APRV extracted from AA23 cells. Lane2: dsRNA of APRV extracted from C6/36 cells, dsRNA of APRV extracted from A20 cells.

In order to rule out the presence of any 10th segment (which might be in a truncated form), gels were overloaded with genome extract; however, this failed to identify any band other than the 9 bands constituting the genome.

Virus replication in mosquito cells

The viral genome was visible in RNA extracts (Fig. 1) AA23, C6/36 cells and A20 cells that were infected with AP61 lysate.

The extracts of AE and Aw-albus cells incubated with AP61 lysate (or with virus purified from infected AA23) showed no visible poly-segmented dsRNA profiles. These extracts were also negative by RT-PCR which showed that these cell lines do not support the replication of aedes pseudoscutellaris reovirus.

The extract of AP61 cells showed no visible poly-segmented dsRNA profiles but were positive by RT-PCR analysis suggesting that these cells contained low amounts of the virus genome (see below).

Virus presence in AP61 cells from various sources

All three AP61 cell lines were found to contain the aedes pseudoscutellaris reovirus. PCR using aedes pseudoscutellaris reovirus segments 6 and 7 specific primers produced amplicons with the expected sizes. The products were sequenced and their sequence was found to be 100% identical. Amplicons were only detectable using a nested PCR assay. The number of viral genome copies per cell was calculated using 'Taqman' quantitative PCR methodology as between 1 and 5.

Effect of 2-aminopurine on virus replication in AP61 cells

The cells treated with 2-aminopurine (2-AM) showed an increase in the number of viral particles. The quantification by Taqman has shown that 60–80 APRV genomes could be estimated per AP61 cell. This represented an increase of over 10-folds in the initial number of viral genome copies estimated by the same methodology.

Virus replication in mammalian cells and in mice

Extracts of mammalian cells from the three passages in mice, failed to generate any cDNA products, using the primers specific to APRV segments 6 and 7. The RNA extracted from blood of the injected mouse also remained negative for APRV (by RT-PCR) from day 0 to day 12 post-injection.

Electron microscopy

Purified APRV particles analyzed by TEM showed a morphology typical of the cores of turreted reoviruses (Fig. 2). In particular, the morphology of APRV was similar to that observed for cypoviruses (Mertens et al., 2005; Hill et al., 1999). The mean diameter of the particle is approximately 49–50 nm with a central part that is 36–37 nm. Turrets were visible projecting from the particle surface.

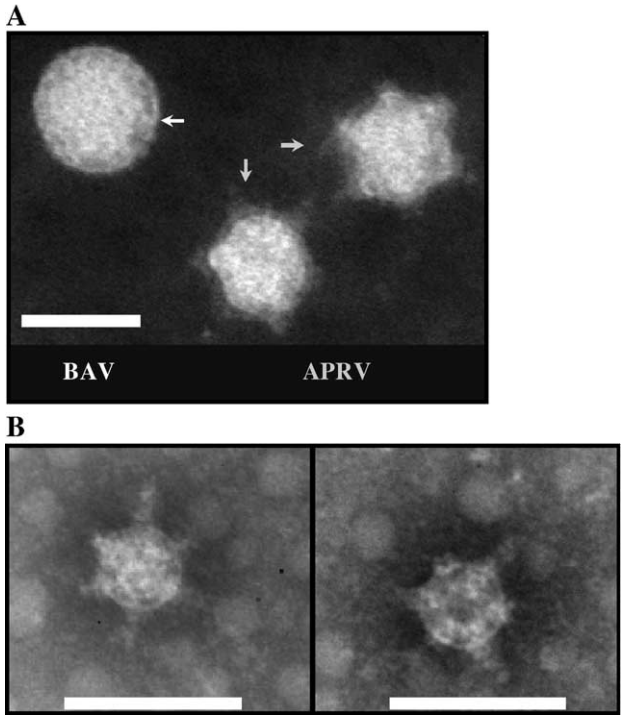

Fig. 2. Electron micrographs of purified APRV particles. (A) Electron micrograph showing 2 purified APRV particles (right) stained with potassium phosphotungstate and one core particle of the non-turreted Banna virus (on the left for the purpose of comparison). (B) Electron micrograph of APRV showing the turrets extending above the particle surface. The scale bar represents 50 nm.

Physicochemical properties of aedes pseudoscutellaris reovirus particles

The virion infectivity was not affected by treatment with 1% deoxycholate but was totally destroyed after 0.1% SDS treatment. Repeated treatment with Freon 113 or Vertrel XF did not affect the infectivity.

Freezing the virus at −80 °C or at −20 °C destroyed infectivity, even in the presence of 50% FBS. However, the virus is stable at 4 °C for at least 5 months and at room temperature for up to 3 weeks. Heating to 50–60 °C for 30 min also abolished infectivity.

Infectivity was retained at pH values between 6 and 8. Between pH 4 and 5 or between pH 9 and 10, the infectivity is reduced by a factor of 10^{-1}. The virion morphology was considerably distorted at pH lower than 5 and virions were completely disrupted at pH lower than 3.5.

Sequence analysis and comparison

Segments 1 to 9 of APRV were analyzed and their sequences deposited in GenBank under accession numbers (DQ08276–DQ08284). The positive strands of all nine segments of the APRV genome have conserved sequences in the 5′ and 3′ non-coding regions (NCR) (5′-AGUU$^{A}/_{U}$AA$^{A}/_{C}^{A}/_{C}$— — — —$^{U}/_{G}$UUnnn$^{C}/_{U}$nn$^{A}/_{U}$AGU-3′, where *n* = any nucleotide; Table 1). Comparisons of these conserved termini with those of member-viruses of the genera *Cypovirus*, *Oryzavirus* and *Fijivirus* (Table 1) showed that only the first 3 nucleotides

Table 1
Lengths of dsRNA segments 1 to 9, encoded putative proteins, conserved sequences at the 5′ and 3′ non-coding regions of aedes pseudoscutellaris reovirus

	Accession number	Segment length (bp)	Proteins		5′ NCR		3′ NCR	
			Length	Mass* (Da)	Length (bp)	Terminal sequences	Terminal sequences	Length (bp)
Segment 1	DQ08276	3817	1189	134709	181	5′-AGUUUAAUUCCC------	------UUUGAUCCUAAGU-3′	69
Segment 2	DQ08277	3752	1233	143306	27	5′-AGUUAAACCGCC------	------UUUGUUUUUAAGU-3′	26
Segment 3	DQ08278	3732	1202	136274	33	5′-AGUUUAAAACCC------	------UUUUGAUACUAGU-3′	93
Segment 4	DQ08279	3375	1003	116502	24	5′-AGUUUAAAAACC------	------UUUAAUCCUAAGU-3′	101
Segment 5	DQ08280	3227	1056	121241	16	5′-AGUUAAAACCAC------	------GUUUAGUAAUAGU-3′	43
Segment 6	DQ08281	1775	540	62225	23	5′-AGUUUAAACCCA------	------UUUUGAUACUAGU-3′	132
Segment 7	DQ08282	1171	348	39441	22	5′-AGUUAAAAAACCA------	------UUUUAGUAAUAGU-3′	105
Segment 8	DQ08283	1151	345	39552	16	5′-AGUUUAAAUCCC------	------UUUUGAUAAUAGU-3′	100
Segment 9	DQ08284	1147	278	32181	89	5′-AGUUAAAAACCCA------	------GUUUAGUAGUAGU-3′	224
					Consensus	5′-AGUU$^{A}/_{U}$AANNN$^{A}/_{C}$$^{A}/_{C}$------$^{U}/_{G}$UUNNN$^{C}/_{U}NN^{A}/_{U}$AGU-3′		

Genus	Species	5′ end 3′ end
Dinovernavirus	APRV	5′-AGUU$^{A}/_{U}$---------- $^{A}/_{U}$AGU-3′
Cypovirus	CPV1	5′-AGUAAA----------GUUAGCC-3′
	CPV2	5′-AGUUU----------GAGUUUGC-3′
	CPV15	5′-AUUAAAAA---------- GC-3′
Fijivirus	CPV4	5′-AAUCGACG----------GUCGUAUG-3′
	NLRV	5′-AGU----------GUUGUC-3′
	MRCV	5′-AAGUUUUUU---------- GUC-3′
	FDV	5′-AAGUUUUUU---------- GUC-3′
Oryzavirus	RBSDV	5′-AAGUUUUUU---------- GUC-3′
	RRSV	5′-GAUAAA---------- GUGC-3′

(A) Mass*: calculated theoretical molecular mass. (B) At the 5′ end, the first three nucleotides (AGU) are conserved between cypoviruses (CPV), fijiviruses (NLRV: *Nilaparvata lugens reovirus*, MRCV: *Mal de Rio Cuarto virus*, FDV: *Fiji disease virus*, RBSDV: *Rice black streak dwarf virus*) and APRV (boxed). At the 3′ end, notice that there is no conservation between APRV and the other genera. However, the last two nucleotides in oryzaviruses (GC) seem to be conserved with those of certain cypoviruses.

in the 5′ NCR (AGU) are conserved between APRV, cypoviruses and NLRV (*Nilaparvata lugens reovirus*, *Fijivirus*). The 3′ termini of APRV differs from those of the cypoviruses, NLRV and RRSV (*Rice ragged stunt virus*, *Oryzavirus*).

The first and last nucleotides of the APRV genome segments are complementary (A and U), by contrast to those of cypoviruses and fijiviruses which are not. The mean G + C content of the APRV genome is 34.4%, compared to 34.8% for NLRV, 44.7% for RRSV and 43% for the cypoviruses.

Sequence comparison to other viruses, particularly those of family Reoviridae, has shown significant aa identities (in all proteins encoded by the genome), with other reoviruses.

Members of the genera *Cypovirus*, *Oryzavirus* and *Fijivirus*, which are also turreted, showed the highest aa identities (19–31%—partial sequences: Fig. 3 and Table 2). However, weaker aa identities were also detected with other proteins belonging to members of the genera of other turreted viruses such as *Mycoreovirus*, *Coltivirus* and *Orthoreovirus* as shown in Table 2. Interestingly, the protein encoded by segment 8 (VP8) of APRV showed a partial match (aa: 131–260, 27% identity) to a non-turreted virus protein namely the NSP3 of rotavirus A (RvA). NSP3 of RvA has been found to be involved in translational regulation and host cell shut-off through binding to eIF4G1 factor (Deo et al., 2002; Groft and Burley, 2002).

It is interesting to note that the VP1 (1189 aa long) of APRV showed a significant match (22% aa identity, with a probability of $2e^{-6}$) over a sequence between aa 756 and aa 1173 (a 418 aa-long fragment) to a minor structural protein (accession number AAC60740) of a ssDNA parvo-like virus isolated from the silkworm (Bando et al., 1995). Observation of significant similarities between dsRNA viruses and DNA viruses is rather unusual. The VP3 of APRV also showed some similarity to the histidine kinase of Heliothis zea virus (accession number AAN04301: aa 256–544, identity 18%).

Therefore, putative functions of VP1 to VP9 of APRV were proposed based on the identified similarities, as shown in Table 2. These comparisons, particularly with cypoviruses, fijiviruses and oryzaviruses, seem to suggest that there is no equivalent to polyhedrin gene present in cypoviruses and to confirm the status of APRV as an authentic nine-segmented virus that is distinct from the cypoviruses.

Phylogenetic analysis

The virus RNA-dependent RNA polymerase (RdRp) of member viruses of family Reoviridae is considered as the only target gene which allows relevant phylogenetic analysis within this family (Attoui et al., 2002). It has been suggested as a general rule that a value lower than 30% aa identity among RdRps distinguishes genera (Attoui et al., 2002). However, two exceptions to this rule have been discussed: the case of aquareoviruses and orthoreoviruses which clearly have a common ancestor with an aa identity of 42% and that of rotavirus B which is only 22% identical to other rotaviruses.

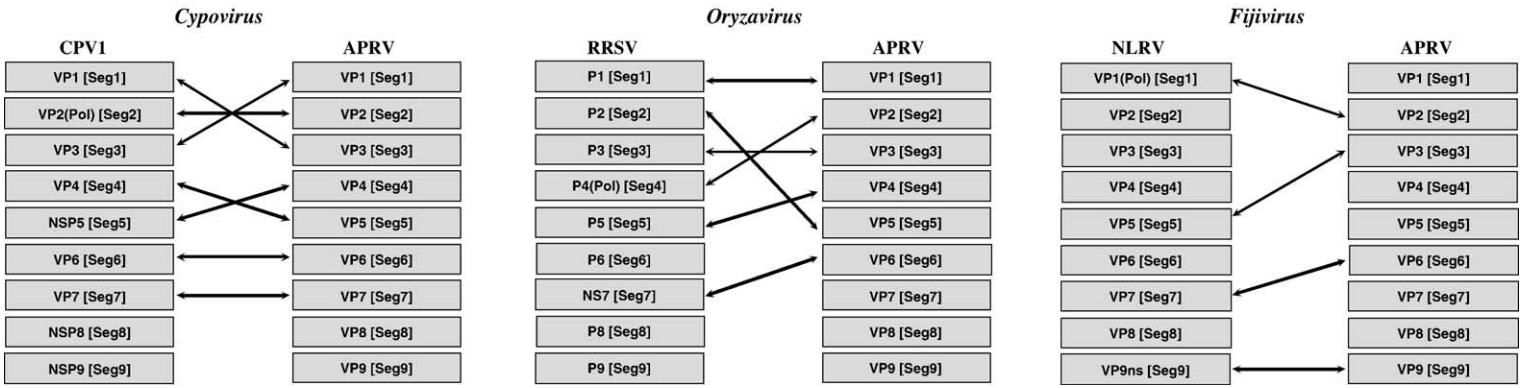

Fig. 3. Correspondence between aedes pseudoscutellaris reovirus (APRV) and cypovirus or oryzavirus or fijivirus. Pol: RNA-dependent RNA polymerase.

Table 2
Amino acid identities between APRV proteins and other Reoviridae proteins

APRV/function by similarity	CPV1 (position) [%]	RRSV (position) [%]	NLRV (position) [%]	CpMYRV-1 (position) [%]	CTFV (position) [%]	MRV3 (position) [%]
VP1/possible cell attachment	VP3 (768–1157) [23]	P1 (963–1208) [22]				σ1 (44–231) [25]
VP2[a]/RdRp	VP2 (23–1206) [26]	P4 (68–1030) [23]	VP1[a] (599–624) [22]	VP1[a] (62–877) [20]		
VP3/T2	VP1 (6–1302) [21]	P3 (398–645) [21]	VP5 (339–698) [21]			
VP4/non-structural	NSP5 (795–874) [28]	P5 (727–789) [31]				
VP5/possible capping enzyme	VP4 (38–1045) [22]	P2 (589–1102) [19]				
VP6/possible NTPase	VP6 (1–555) [22]	NS7 (399–591) [23]	VP7 (399–591) [23]	VP6 (413–446) [43]	VP10 (345–491) [25]	
VP7/non-structural	VP7 (17–85) [27]					
VP8/possible translational regulation						
VP9/Viral inclusion bodies			VP9 (169–218) [31]			

The AA identities calculated between putative aedes pseudoscutellaris reovirus proteins and other Reoviridae members are given between brackets. Values between parenthesis are the positions of aa. CPV1: *Cypovirus type 1* (*Cypovirus*); RRSV: *Rice ragged stunt virus* (*Oryzavirus*); NLRV: *Nilaparvata lugens reovirus* (*Fijivirus*); RvA: *Rotavirus A* (*Rotavirus*); CpMYRV-1: *Mycoreovirus 1* (*Mycoreovirus*); CTFV: *Colorado tick fever virus* (*Coltivirus*); MRV3: *Mammalian orthoreovirus* serotype 3 (*Orthoreovirus*).
[a] Virus RdRp.

The sequence comparison clearly showed that aedes pseudoscutellaris reovirus is related to cypoviruses, fijiviruses and oryzaviruses. AA identity in the RdRp gene is 26, 23 and 22%, respectively (Table 2). Therefore, the newly identified aedes pseudoscutellaris reovirus belongs to a new separate genus. However, RdRp gene analysis suggests a common ancestry between these 4 groups of viruses. Sequence alignment showed that the most conserved region among the polymerases lies within the core domain of the enzyme (located at similar positions in RdRps of different reoviruses) between aa 634 and 761 of APRV. This region and the whole of the polymerase sequence were used in phylogenetic comparisons (Figs. 4A and B, respectively). Evolutionary relationship between aedes pseudoscutellaris reovirus, cypoviruses, fijiviruses and oryzaviruses was further confirmed by homology depicted in other genes (see Fig. 4 and Table 2).

Discussion

Insect and tick cell lines have been used in routine isolation of arboviruses. Many insect or tick cells have been found to contain endogenous viruses, infecting the cell line in a persistent manner. Examples are the *Aedes albopictus* C6/36 cells (Jousset et al., 1993) containing a densovirus (ssDNA), the *A. pseudoscutellaris* AP61 cells containing also a densovirus (Gorziglia et al., 1980) and the *Ixodes scapularis* IDE2 cells containing an orbivirus (Attoui et al., 2001) designated *St. Croix river virus* (dsRNA). Moreover, C6/36 cells and other mosquito cells were found to contain a genome of a flavivirus under a DNA form, integrated in the cell chromosomes (Crochu et al., 2004).

We have identified a dsRNA virus that infects persistently the *A. pseudoscutellaris* AP61 cell line. *A. pseudoscutellaris* mosquitoes are known to be confined to the south pacific region (Marks, 1954). AP61 cells were obtained from 3 independent sources and the presence of the virus was confirmed in each culture. This virus was designated aedes pseudoscutellaris reovirus. The negative staining electronmicroscopy showed that aedes pseudoscutellaris reovirus is single-shelled with a morphology similar to that of cypoviruses. However, by contrast to cypoviruses, aedes pseudoscutellaris reovirus is non-occluded. The characterization of the dsRNA genome has shown that aedes pseudoscutellaris reovirus genome is composed of 9 dsRNA segments with a total length 23,355 bp.

We shall discuss hereunder the taxonomic status of aedes pseudoscutellaris reovirus. Despite the fact that its dsRNA genome is made of 9 segments (while Reoviridae genomes by definition contain 10–12 segments), APRV is clearly a member of family Reoviridae. First, its polymerase is significantly related to those of cypoviruses, oryzaviruses and fijiviruses, and homology can also be detected in various other genes. Second, the morphology of the virus particle is similar to that of turreted cores of members of the family and to cypoviruses. Third, other general features including the size of the virion, the length of the genome and the physico-chemical properties are similar to those of members of this family.

To date, insect reoviruses have been classified into two genera: (i) the occluded viruses belong to genus *Cypovirus*, and are characterized by missing capsid protein layers (Mertens et al., 2005; (ii) the non-occluded viruses have been recently classified within genus *Idnoreovirus* and include 10-segmented viruses from *Drosophila melanogaster*, *Ceratitis capitata*, *Hyposoter exiguae* and *Diadromus pulchellus* (Mertens et al., 2005). However, aedes pseudoscu-

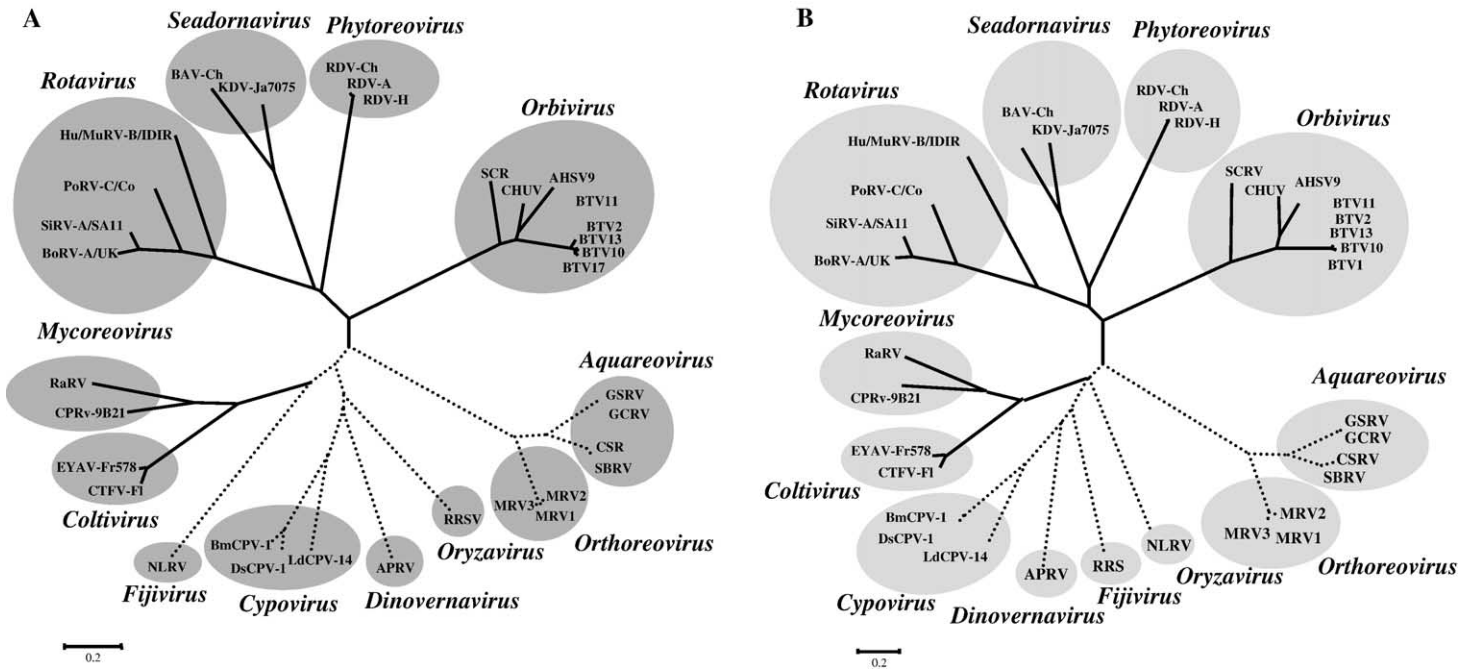

Fig. 4. Neighbor-joining tree built with the available sequences of RdRps of representative members of family Reoviridae. The accession numbers and abbreviations are those used in Table 2. (A) Tree built with partial sequences of the polymerases (aa 634 and 761 relative to APRV polymerase). (B) Tree built with full-length sequences of the polymerases.

tellaris reovirus cannot be convincingly assigned to either of these two genera. The purified aedes pseudoscutellaris reovirus particle morphology is highly similar to that of cypoviruses; however, APRV particles are found naturally non-occluded by contrast to cypoviruses. In addition, the genetic relationship between aedes pseudoscutellaris reovirus and cypoviruses is that existing between distinct genera and comparable to the genetic relationship between aedes pseudoscutellaris reovirus and oryzaviruses or aedes pseudoscutellaris reovirus and fijiviruses. Aedes pseudoscutellaris reovirus possesses conserved terminal regions in all 9 segments. The 3′NCR does not show conservation with that of cypoviruses while the first 3 nucleotides (AGU) in the 5′ NCR are conserved among aedes pseudoscutellaris reovirus and cypoviruses. However, they are also conserved between aedes pseudoscutellaris reovirus and fijiviruses (www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/CPV-RNA-Termin.htm). The G + C content (34.4%) of aedes pseudoscutellaris reovirus genome is comparable to that of fijiviruses (34.8%) and lower than those of oryzaviruses (44.7%) and cypoviruses (43%). Accordingly, genetic analysis does not provide evidence that aedes pseudoscutellaris reovirus belongs to genus *Cypovirus*. Regarding the relationship to idnoreoviruses, these viruses have a different morphology (triple layered particles) and genetic analysis shows only 8% aa identity with the RdRp gene of Diadromus pulchellus reovirus (DPRV, *Idnoreovirus,* Renault et al., 2005). Altogether, these arguments exclude the belonging of aedes pseudoscutellaris reovirus to genus *Idnoreovirus*.

Our phylogenetic analysis including representative members of the different genera of family Reoviridae does not suggest that aedes pseudoscutellaris reovirus could be classified as a member of any of these genera. Accordingly, data provided in this work make aedes pseudoscutellaris reovirus the first representative of a new taxonomic group of dsRNA viruses, suggesting that it should be recognized as the type species within a distinct new genus of the family Reoviridae. This genus was designated Dinovernavirus (sigla from D: Double-stranded, i: insect, nove: nine from the latin "novem" for the 9 segments, rna: RNA, virus). It is the first genus within family Reoviridae with a reported representative member having a nine-segmented dsRNA genome, therefore expanding the definition of the family to viruses with 9–12 dsRNA genome segments. A proposal to recognize the new genus of nine-segmented viruses within family Reoviridae was made to the ICTV meeting in San Francisco, July 2005. It received initial support and was passed for public consultation.

The discovery of this new genus and its relation to cypoviruses, oryzaviruses and fijiviruses, following the reported relationship between orthoreoviruses and aquareoviruses (Attoui et al., 2002) or between coltiviruses and mycoviruses (Suzuki et al., 2004) provides increasing evidence that the evolutionary origin of the different genera within family Reoviridae is not polyphyletic. This might represent a starting point for a possible reconstruction of the evolutionary history of this family.

Materials and methods

Cell cultures

AA23 (*A. albopictus*) cells were grown in a mixture of 1 part of Mitsuhashi/Maramorosh insect medium and 1 part of Schinder's insect medium supplemented with 10% fetal bovine serum (FBS) at 28 °C. C6/36 (*A. albopictus*) cells, A20 and AE cell lines (both from *Aedes aegypti*), AP61 (*A. pseudoscutellaris*) cells and Aw-albus (*Aedes w-albus*) were grown in Leibovitz's L-15 medium supplemented with 10% FBS at 28 °C.

The AP61 cells were obtained from 3 different sources (Dr. J.-P. Durand: IMTSSA virology unit, Marseilles, France; Pr. E.A. Gould: Institute for ecology and hydrology, Oxford, UK; Pr. R. B. Tesh: center for tropical diseases at UTMB, Texas).

The mammalian cells L929 (mouse fibroblast), BHK-21 (hamster kidney), Vero (monkey kidney), BGM (monkey kidney), Hep-2 (human cervix cancer) and MRC-5 (human lung fibroblasts) were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS at 37 °C under CO_2.

Virus propagation, purification and electron microscopy

AP61 cells (25 cm^2 flask) were grown to confluence and pelleted at 2000 g. The cells were lysed in deionized water (18 MΩ) and the lysate was buffered with serum-free L-15 medium. 200 μl of the lysate suspension was incubated for 1 h at 28 °C with a monolayer of AA23 cells (25 cm^2 flask) and 7 ml of complete L-15 was subsequently added. The culture was incubated at 28 °C for 5 days. 200 μl of the supernatant of this culture was used to re-infect fresh AA23 cultures and 3 passages in AA23 cells were performed. APRV was then plaque purified from the supernatant of the 3rd passage in AA23 cells overlaid with seaplaque agarose and plaques were identified by staining with Trypan blue. The plaque purification process was repeated 3 times.

The virus was further propagated in 175 cm^2 culture flasks, and infected cells were harvested at day 7 post-infection (pi). The cells and supernatant were centrifuged at 3000 × *g* and the supernatant was recovered. Virus from supernatant was concentrated by centrifugal ultrafiltration using PES ultrafiltration units with 5000 MWCO (Vivasciences).

Cells were lysed by treatment with 18 MΩ deionized water and mixed with the virus from supernatant. The suspension was made 100 mM Tris–HCl pH 7.5. The mixture was treated with an equal volume of Vertrel XF, centrifuged at 2000 × *g* and the supernatant was recovered, layered on top of a discontinuous Optiprep gradient (10, 20, 30 40 and 50% optiprep in 100 mM Tris–HCl pH 7.5) and centrifuged for 2 h at 21,0000 × *g* in an SW41 rotor (Mohd Jaafar et al., 2005b). The light blue virus band was recovered at the interface of the 40 and 55% layers.

The virus was adsorbed to formvar/carbon coated grids, stained with 2% potassium tungstate for 30 s and dried prior to

being examined by electron microscopy on a Philips Morgagni 280 transmission electron microscope (TEM).

Assay of aedes pseudoscutellaris reovirus replication in various mosquito and mammalian cells

The virus was inoculated into insect cell lines, namely C6/36, A20, AE and Aw-albus. It was also inoculated into the mammalian cells L929, BHK-21, Vero, BGM, Hep-2 and MRC-5. For this purpose, 100 μl of the supernatant of APRV-infected AA23 culture was added to the cell monolayers (25 cm^2) and incubated at 28 °C for the mosquito cell lines and at 37 °C (or 33 °C) for the mammalian cell lines for 1 h. The cells were washed twice with PBS and the culture medium was added. At day 5 pi, the cells were lysed with deionized water, mixed with the supernatant and used to re-infect new cells. This was repeated for 3 passages. In each passage, the RNA was extracted from cells and supernatant at day 5 pi (see below) and processed for agarose gel electrophoresis and/or RT-PCR using specific APRV primers as described below.

Isolation and purification of nucleic acids

APRV dsRNA was extracted from infected cells using a commercially available guanidinium isothiocyanate based procedure (RNA NOW reagent: Biogentex, TX, USA). Briefly, cells from culture flasks were scraped off and pelleted by centrifugation at 800 × *g* at 4 °C for 10 min. The pellet was dissolved in 500 μl of "lysis reagent: 6 M guanidinium isothiocyanate" then mixed with 500 μl of the extraction reagent. Two hundred microliters of chloroform was added and the mixture was shaken for 1 min, kept for 10 min on ice, then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was recovered, mixed with 900 μl of 100% isopropanol and incubated overnight at −20 °C. The RNA was pelleted by centrifugation at 18,000 × *g*, for 10 min at 4 °C, washed with 75% ethanol, dried and dissolved in water. The dsRNA was further purified by precipitating high molecular weight ssRNAs in 2 M LiCl, as described elsewhere (Attoui et al., 2000).

Nature of the poly-segmented genome

The extracted viral genome was treated with nucleases to verify the nature of the nucleic acids. Briefly, 2 μg of the genome extract was incubated with 100 units of RNase-free DNase I (Invitrogen) for 1 h at 37 °C to verify if the genome contained any DNA. Similarly, 2 μg of the genome was incubated with 5 μg/ml of RNase A (Roche) in 2× SSC and in 0.1× SSC medium (1× SSC: 0.015 M sodium citrate and 0.15 M NaCl).

The RNA extract from infected cells was run on a 1% agarose gel in TAE buffer or 10% polyacrylamide gel in tris–glycine buffer as described by Laemmli (1970).

The bands separated by PAGE were assayed by densitometry using the BioDocAnalyze software (Biometra) in order to assay the number of copies per separated band.

RT-PCR of the RNA extract from the tested cell lines

The dsRNA was copied into cDNA using random hexanucleotide primers as previously described (Attoui et al., 2000). Briefly, the dsRNA was denatured in 15% DSMO by heating at 100 °C for 1 min and incubated immediately on ice. The reverse transcription was realized using the Superscript III reverse transcriptase (Invitrogen) at 42 °C.

The resulting cDNA was PCR amplified using primers designed from segments 6 and 7. The sequences and positions of these primers are shown in Table 3.

Assay of aedes pseudoscutellaris reovirus replication in mice

Ten-week-old mice were inoculated intraperitoneally with 200 pfu of APRV. Blood was recovered from the caudal vein at days 0, 3, 6, 9 and 12. The blood samples were frozen at −20 °C. The frozen blood was extracted using the RNA NOW reagent (Biogentex) as described by the manufacturer and processed for RT-PCR using specific APRV primers as described below.

Physicochemical properties of aedes pseudoscutellaris reovirus particles

The virion infectivity was assayed following treatment with detergents including 1% sodium deoxycholate as described earlier (Borden et al., 1971) and 0.1% SDS. Treatment with Freon 113 or its ozone-friendly replacement 2,3-dihydrodecafluoropentane known as Vertrel XF (Dupont) was also assayed.

The effect of temperature (over a range from −80 °C to + 60 °C) and of pH (pH 3 to pH 10) on the infectivity was assayed.

Quantification of the virus in AP61 cells

The virus titer in AP61 cells was assayed by RT-PCR Taqman methodology, using the ABI PRISM 7000 Sensor detection system (ABI). The primers and probes were designed from the sequence of segment 2. The forward primer was 5′-TCATCACACGCAAATGTTGACTT-3′ (position: 2010–2032), the reverse primer was 5′-GCTATTGCTATACTT-TTCCCGAATTC-3′ (position: 2093–2068) and the probe was 5′-ACTAATAGAAACTGCAGCAACTACATTCAC-TAA-3′ (position: 2034–2066).

Cells were counted using Neubauer hemocytometer, lysed with deionized water and treated with 5 μg/ml of RNAse A at 37 °C for 30 min to remove ssRNA. Virus RNA was extracted using RNA NOW as described above, denatured at 100 °C for 4 min and reverse transcribed using random hexaprimers and Superscript III enzyme. As a standard for the determination of the copy number, segment 2 was purified on gel and quantified by UV spectrophotometry.

Effect of 2-aminopurine on virus replication in AP61 cells

2-Aminopurine (2-AM) is known to inhibit the dsRNA-inducible protein kinase (PKR) in mammalian cells, therefore

Table 3
First round and nested PCR primers designed from the sequences of segments 6 and 7 of APRV

Primer name	Specificity	Sequence (5′→3′)	Position	Orientation	Amplicon size in bp
Api6S1	Segment 6	GAAGCACATACCATCAGAGATAGC	452–475	Sense	485
Api6R1	Segment 6	GGTCATCAAATACACTGGCAACGC	936–913	Anti-sense	
Api6S2	Segment 6	AAGTCGTGTTGATTAATAAGACAGG	619–643	Sense	250
Api6R2	Segment 6	TTCATTACTGGTACGCCATGCACG	868–845	Anti-sense	
Api7S1	Segment 7	CCGGAGATACCAGTCTACCAGAC	312–334	Sense	406
Api7R1	Segment 7	TCTCCATATTCCGAGTCTCAACG	717–694	Anti-sense	
Api7S2	Segment 7	ATCTAGTACGTCAGCCACCTATGG	384–407	Sense	250
Api7R2	Segment 7	TCCTCGTTTGCGTCGACATATTCC	633–610	Anti-sense	

enhancing replication of dsRNA viruses (Strong et al., 1998). Insect cells, such as *D. melanogaster*, were found to contain a homologue to PKR identified as PERK (PKR-like ER kinase) which specifically phoshorylates eIF2α factor (Pomar et al., 2003). Subconfluent monolayers of AP61 cells were incubated in the presence of L-15 culture medium with or without 5

Table 4
Sequences used in RdRps phylogenetic analysis of aedes pseudoscutellaris reovirus: the abbreviations listed are those used in Fig. 4

Species	Isolate	Abbreviation	Accession number
Genus *Seadornavirus* (12 segments)			
Banna virus	Ch	BAV-Ch	AF168005
Kadipiro virus	Java-7075	KDV-Ja7075	AF133429
Genus *Coltivirus* (12 segments)			
Colorado tick fever virus	Florio	CTFV-Fl	AF134529
Eyach virus	Fr578	EYAV-Fr578	AF282467
Genus *Orthoreovirus* (10 segments)			
Mammalian orthoreovirus	Lang strain	MRV-1	M24734
	Jones strain	MRV-2	M31057
	Dearing strain	MRV-3	M31058
Genus *Orbivirus* (10 segments)			
African horse sickness virus	Serotype 9	AHSV-9	U94887
Bluetongue virus	Serotype 2	BTV-2	L20508
	Serotype 10	BTV-10	X12819
	Serotype 11	BTV-11	L20445
	Serotype 13	BTV-13	L20446
	Serotype 17	BTV-17	L20447
Palyam virus	Chuzan	CHUV	Baa76549
St. Croix river virus	SCRV	SCRV	AF133431
Genus *Rotavirus* (11 segments)			
Rotavirus A	Bovine strain UK	BoRV-A/UK	X55444
	Simian strain SA11	SiRV-A/SA11	AF015955
Rotavirus B	Human/murine strain IDIR	Hu/MuRV-B/IDIR	M97203
Rotavirus C	Porcine Cowden strain	PoRV-C/Co	M74216
Genus *Aquareovirus* (11 segments)			
Golden shiner reovirus	GSRV	GSRV	AF403399
Grass Carp reovirus	GCRV-873	GCRV	AF260511
Chum salmon reovirus	CSRV	CSRV	AF418295
Striped bass reovirus	SBRV	SBRV	AF450318
Genus *Fijivirus* (10 segments)			
Nilaparvata lugens reovirus	Izumo strain	NLRV-Iz	D49693
Genus *Phytoreovirus* (10 segments)			
Rice dwarf virus	Isolate China	RDV-Ch	U73201
	Isolate H	RDV-H	D10222
	Isolate A	RDV-A	D90198
Genus *Oryzavirus* (10 segments)			
Rice ragged stunt virus	Thai strain	RRSV-Th	U66714
Genus *Cypovirus* (10 segments)			
Bombyx mori cytoplasmic polyhedrosis virus 1	Strain I	BmCPV-1	AF323782
Dendrlymus punctatus cytoplasmic polyhedrosis 1	DsCPV-1	DsCPV-1	AAN46860
Lymantria dispar cytoplasmic polyhedrosis 14	LdCPV-114	LdCPV-114	AAK73087
Genus *Mycoreovirus* (11 or 12 segments)			
Rosellinia anti-rot virus	W370	RaRV	AB102674
Cryphonectria parasitica reovirus	9B21	CPRV	AY277888

mM 2-AM. The RNA was extracted at 24, 48 and 72 h post-addition of 2-AM and analyzed by gel electrophoresis, RT-PCR and quantitative Taqman RT-PCR.

Cloning of the dsRNA segments

The genome segments of APRV were copied into cDNA, cloned and sequenced as previously described (Attoui et al., 2000). Briefly, a defined 3′-amino blocked oligodeoxyribonucleotide was ligated to both of the 3′ ends of the dsRNA segments, using T4 RNA ligase, followed by reverse transcription and PCR amplification using a complementary primer. PCR amplicons were analyzed by agarose gel electrophoresis, ligated into the PGEM-T cloning vector (Promega) and transfected into competent XL-blue *E. coli*. Insert sequences were determined using M13 universal primers, the D-Rhodamine DNA sequencing kit and an ABI prism 377 sequence analyzer (Perkin Elmer).

Sequence analysis

All sequence alignments were performed using the Clustal W software program (Thompson et al., 1994). Phylogenetic analyses were carried out with the software program MEGA3 (Kumar et al., 2004) using the p-distance determination algorithm. Sequence relatedness is reported as percentage identity.

Analysis of APRV sequence data was performed with a local BLAST program implemented in the DNATools package (version 5.2.018, S.W. Rasmussen, Valby data center, Denmark), using a database built from all available sequences of virus-members of family Reoviridae.

The RNA polymerase sequence of APRV was compared with the sequences of RNA-dependent RNA polymerases of representative strains of viruses representing the genera of the family Reoviridae. GenBank accession numbers are provided in Table 4.

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