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Proteomic analysis of *Chilo iridescent virus*

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ABSTRACT

In this first proteomic analysis of an invertebrate iridovirus, 46 viral proteins were detected in the virions of *Chilo iridescent virus* (CIV) based on the detection of 2 or more distinct peptides; an additional 8 proteins were found based on a single peptide. Thirty-six of the 54 identified proteins have homologs in another invertebrate and/or in one or more vertebrate iridoviruses. The genes for 5 of the identified proteins, 22L (putative helicase), 118L, 142R (putative RNaseIII), 274L (major capsid protein) and 295L, are shared by all iridoviruses for which the complete nucleotide sequence is known and may therefore be considered as iridovirus core genes. Three identified proteins have homologs only in ascoviruses. The remaining 15 identified proteins are so far unique to CIV. In addition to broadening our insight in the structure and assembly of CIV virions, this knowledge is pivotal to unravel the initial steps in the infection process.

Introduction

Chilo iridescent virus (CIV), also known as *Invertebrate iridescent virus 6*, belongs to the family *Iridoviridae* and is the type species of the genus *Iridovirus* (Fauquet et al., 2005; Williams, 1996; Williams et al, 2005; Willis, 1990). Iridoviruses are large, cytoplasmic, icosahedral viruses with a linear double-stranded DNA genome, which is both circularly permuted and terminally redundant (Darai et al., 1983; Goorha and Murti, 1982). The CIV virion consists of an unusual three layer structure containing an outer proteinaceous capsid, an intermediate lipid membrane, and a core DNA–protein complex containing the 212, 482 bp genome (Jakob et al., 2001; Williams, 1996; Williams et al., 2005). Up to now, thirteen complete sequences of iridovirus genomes have been published, including CIV (Huang et al., 2009; Williams et al., 2005). The availability of the CIV sequence facilitates the identification and functional analysis of the proteome of CIV virions. Replication of CIV occurs in the nucleus of infected cells and the assembly takes place in the cytoplasm (Goorha and Murti, 1982).

Many questions remain to be answered concerning the structure and scaffolding of the virus particles, the nature of virus–host interactions and the initial steps in virus infection, including the mechanism behind the onset of transcription of CIV genes. Viral structural proteins are likely to play crucial roles in these processes. Initiation of viral transcription for instance requires one or more virion proteins, since CIV DNA alone is not infectious, similar to what has been shown for the vertebrate iridovirus Frog virus 3 (Willis and Granoff, 1985). In previous studies, efforts have been made to characterize the polypeptides in CIV virions by one- or two-dimensional SDS-PAGE. The presence of 21–28 polypeptides was revealed by one-dimensional SDS-PAGE, while 35 polypeptides were observed in two-dimensional SDS-PAGE (Barray and Devauchelle, 1979, 1985; Cerutti and Devauchelle, 1985; Kelly and Tinsley, 1972; Orange and Devauchelle, 1987). The size of these polypeptides ranged from 11 to 300 kDa. However, most of these proteins were not further characterized and it is unknown, except for the major capsid protein MCP, by which CIV genes they are encoded.

In the current study we identified the CIV virion proteins by a proteomic approach, based on a combination of one-dimensional SDS-PAGE and Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC-MS/MS). The data obtained were analyzed by searches against a CIV ORF database. This provided a fast and highly sensitive method for the identification of genes through the sequences of the encoded proteins (Pandey and Mann, 2000).

Results

To identify the virion proteins of CIV, the proteins of purified virion particles were separated by one-dimensional SDS-PAGE. Staining of the gel with colloidal blue revealed at least 21 proteins ranging from 10 to 250 kDa (Fig. 1) much in line to what has been found previously (Barray and Devauchelle, 1979, 1985; Cerutti and Devauchelle, 1985; Kelly and Tinsley, 1972; Orange and Devauchelle, 1987). The gel lane was divided into 6 slices containing proteins with a molecular mass lower than 26 kDa, ranging from 26–34 kDa, 34–43 kDa, 43–55 kDa or 55–95 kDa and higher than 95 kDa, respectively. The proteins were digested with trypsin and analyzed by LC-MS/MS. A decoy database strategy (Elias and Gygi, 2007) was used which, after applying the appropriate filters,

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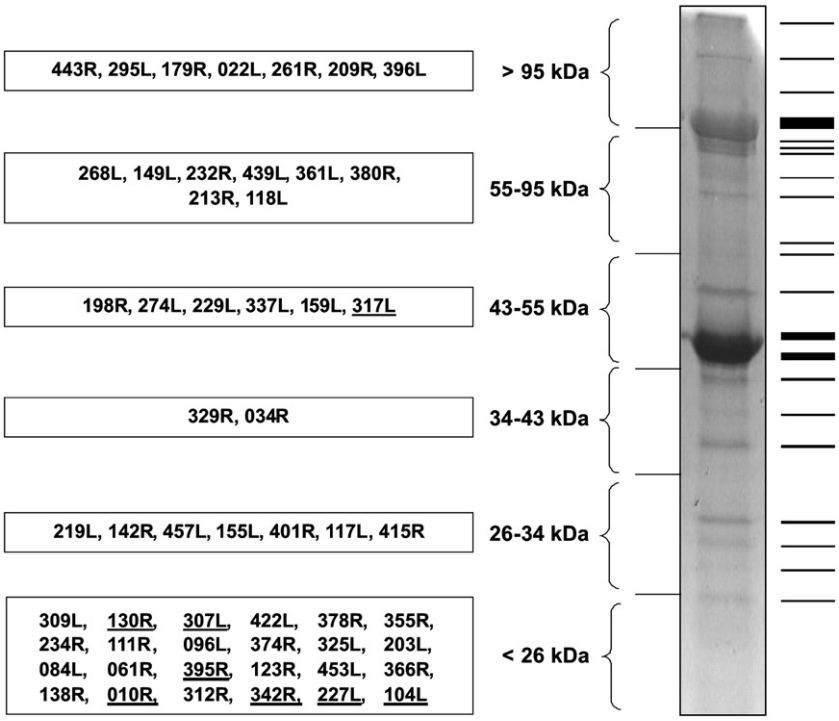

Fig. 1. SDS-PAGE profile and LC-MS/MS identification results of purified CIV virion proteins. CIV proteins were separated by 12% one-dimensional SDS-PAGE and stained with colloidal blue. The SDS-PAGE gel was divided into 6 slices, which, based on comparison to a molecular marker, ranged from higher than 95 kDa, 55–95 kDa, 43–55 kDa, 34–43 kDa, 26–34 kDa and lower than 26 kDa. Proteins were in-gel-digested with trypsin, extracted and subjected to LC-MS/MS. The column on the right indicates the relative abundance of the proteins as visualized by SDS-PAGE. The boxes on the left give the ORF numbers of the identified proteins in a particular gel slice in order of the predicted mass (see Tables 1 and 2). Underlined numbers represent single peptide hits. Indications R and L point towards the direction of transcription from the CIV genome (see Fig. 2).

resulted in 89 protein hits: 54 CIV proteins, 34 contaminants and 1 decoy hit giving a False Discovery Rate of 1.1%. Out of the 54 CIV proteins, 46 of the more abundant proteins were identified with 2 or more peptides (Table 1), while relatively small proteins like ORFs 342R, 227L or 104L as well as some less abundant proteins could be identified with one peptide only (Table 2). The proteins with one hit were manually verified to correlate well to the theoretical b+y ion spectrum and to be unique for one protein only (see also Supplementary Material S1).

The proteins identified are indicated in Fig. 1. A genomic map of CIV ORFs that encode polypeptides represented in the proteome of CIV particles is shown in Fig. 2. For individual CIV virion proteins, 2.7% to 70% of the amino acid sequence was covered with peptides retrieved from the analysis. The major capsid protein (MCP) encoded by ORF 274L is one of the most abundant CIV proteins (Barray and Devauchelle, 1979, 1985; Cerutti and Devauchelle, 1985; Kelly and Tinsley, 1972) and this is clearly reflected by its relative abundance in the current analysis compared to all other CIV proteins (Table 1). The nature of the other major band is not clear at this moment.

Functional domains alluding to possible functions were found in fifteen other identified virion proteins, including three putative serine/threonine kinases (ORFs 209R, 380R and 439R), one dual specificity phosphatase (123R), a protein with homology to the N-terminal domain of viral DNA polymerases (232R), carboxy-terminal domain (CTD) phosphatase (355R), nucleoside triphosphatase (NTP I) (22L), fasciclin (96L), ribonuclease III (142R), tyrosine protein kinase (179R), cathepsin (361L), DNA binding protein (401R), protein disulfide isomerase (453L), lysosome associate membrane glycoprotein (061R), and a ranavirus envelope protein homolog (118L). For the 38 remaining proteins in the virion, we have no clear idea about their specific function at this moment (Tables 1 and 2). Some of these show partial homology to viral proteins of poxvirus, coronavirus or baculovirus origin.

Recent cryoelectron microscopy studies on the capsid of CIV revealed, in addition to MCP, a group of relatively less abundant capsid proteins (Yan et al., 2009). These proteins form a complex which contains a "finger" protein, a "zip" protein, a pentameric complex and an anchor protein. The molecular mass estimations for the finger and zip proteins, the anchor protein and the monomer of the pentameric complex were estimated to be 19.7, 11.9, 32.4 and 39.3 kDa, respectively. For the finger protein the standard deviation was 1.5 kDa, giving a size range of 18.2–21.2 kDa (Yan et al., 2009). Based on this range, seven candidate genes for the finger protein were found in the CIV proteome: ORFs 234R, 111R, 096L, 374L, 325L, 203L, and 084L from large to small (Table 1). The zip protein with an expected size range of 10.5 to 13.3 kDa (1.4 kDa standard deviation) may correspond to three candidate ORFs represented in the proteome: 010R, 138R and 321R. The monomer of the pentameric complex estimated at 39.3 kDa corresponds most closely in size to ORFs 329R and 219L. Anchor protein candidate genes in the CIV proteome could be 457L or 142R, with sizes close to 32.4 kDa (Table 1).

Discussion

The CIV proteome revealed 54 proteins. The genes encoding these virion proteins are scattered over the genome (Fig. 2). It is not known which of the identified proteins are engaged in the scaffolding and assembly of CIV virions, and which are not essential for building the virion structure, but may be important for other aspects, such as the initial stages of the infection process and the regulation of gene expression. It is possible that one of these additional proteins is involved in chaperoning the viral DNA into the nucleus to initiate DNA replication (Willis and Granoff, 1985). To get a better clue about their importance, the conservation of the CIV virion protein genes in the complete genomes of members of the family *Iridoviridae* as well as *Ascoviridae* was assessed. The latter family was included since a common ancestry between iridoviruses and ascoviruses has been inferred from phylogenetic analysis (Stasiak et al., 2000).

Of the 54 ORFs encoding CIV virion proteins identified in the current study, thirty-four have homologs in Invertebrate iridovirus 3 (IIV3), which belongs to the genus *Chloriridovirus* (Table 3, column 2) (Chen

Table 1
Structural proteins of CIV identified by LC-MS/MS with 2 or more distinct peptides. The ORFs are ordered by the mass of the encoded proteins (column 3).

ORF	NCBI accession No	Mol. mass (kDa)	Protein coverage (% by amino acids)	Peptide hits on first rank	Relative abundance[a] (% peak area)	Predicted domains/function
443R	AAK82303	237.22	8.10	15	0.30	
295L	AAK82156	156.42	24.70	43	0.22	Bipartite nuclear localization signal
179R	AAB94478	137.93	14.70	24	0.06	CAP10, Putative lipopolysaccharide-modifying enzyme, tyrosine protein kinase
022L	AAD48148	135.34	32.20	34	0.20	Putative nucleoside triphosphatase I; DEXDc; DEAD-like helicase superfamily
261R	AAK82122	129.06	2.70	30	2.20	Potential repetitive protein
209R	AAK82071	118.34	39.20	52	0.69	Serine/threonine protein kinase
396L	AAK82256	111.28	21.90	29	0.16	Potential repetitive protein
268L	AAK82129	83.22	46.10	74	2.06	
149L	AAB94464	76.41	36.60	72	0.91	
232R	AAK82093	75.56	49.00	75	1.63	DNA polymerase (viral) N terminal domain, 2-cysteine adaptor domain, OTU like cysteine protease
439L	AAK82299	63.45	12.10	8	0.02	Protein kinase domain
361L	AAK82221	60.58	50.70	55	1.06	Peptidase_C1A_CathepsinB
380R	AAK82240	59.91	54.50	73	2.05	S_TKc, Serine or threonine-specific kinase subfamily
213R	AAK82075	58.42	29.70	22	0.16	Putative peptidoglycan bound protein
118L	AAB94444	55.29	55.10	65	1.77	Putative envelope protein
198R	AAK82060	52.15	42.60	26	0.32	
274L	AAK82135	51.29	63.20	157	17.97	Major capsid protein
229L	AAK82090	50.64	22.30	15	0.25	
337L	AAK82199	46.13	27.20	25	0.21	Poxvirus protein of unknown function
159L	AAB94468	45.76	34.90	58	3.98	
329R	AAK82190	42.74	28.80	16	0.29	
219L	AAK82081	34.64	19.00	5	0.01	
142R	AAB94459	33.64	33.60	16	0.25	dsRNA-specific ribonuclease
457L	AAK82317	33.13	25.90	47	2.97	
155L	AAB94465	29.81	39.20	23	0.40	
401R	AAK82261	28.23	25.50	11	0.04	HMG-box superfamily of DNA-binding proteins
117L	AAB94443	27.45	29.70	43	0.93	
415R	AAK82275	26.66	63.20	70	1.34	
309L	AAK82170	24.83	70.00	12	0.10	
422L	AAK82282	22.73	49.50	19	0.20	*Cydia pomonella* granulovirus ORF34
378R	AAK82238	22.21	47.70	12	0.10	2-cysteine adaptor domain
355R	AAK82216	22.01	52.70	10	0.02	Catalytic domain of ctd-like phosphatases
234R	AAK82095	21.09	62.70	63	3.07	
111R	AAB94438	20.01	35.40	9	0.05	
096L	AAB94430	19.69	33.30	11	0.05	Fasciclin domain
374R	AAK82234	19.12	22.40	3	0.00	Bat coronavirus spike protein
325L	AAK82186	18.91	24.50	5	0.08	
203L	AAK82065	18.53	18.80	7	0.02	
084L	AAB94426	18.40	25.50	15	0.04	
061R	AAB94416	17.91	31.60	17	0.01	Lysosome associate membrane glycoproteins
123R	AAB94448	16.38	7.70	3	0.00	Dual specificity phosphatases
453L	AAK82313	15.91	26.10	12	0.05	Protein disulfide isomerase
034R[b]	AAK81969	14.63	16.40	5	0.03	
366R	AAK82226	13.66	17.50	2	0.01	
138R	AAB94455	13.03	16.70	7	0.02	
312R	AAK82173	10.60	20.70	3	0.01	

[a] The relative abundance was calculated by Bioworks as % peak area over all peaks (including contaminants observed) shown after applying the following filter settings: ΔCn>0.08, Xcorr>1.5 for charge state 2+, Xcorr>3.3 for charge state 3+ and Xcorr>3.5 for charge state 4+, Sf>0.6.
[b] This protein was identified in the 34–43 kDa gel piece.

et al., 2008; Song et al., 2004). Fifteen of the 34 ORFs with homologs in IIV3, also have homologs in one or more vertebrate iridoviruses. The CIV proteome shares five ORFs with all iridoviruses: 022L, 118L, 142L, 274L (MCP) and 295L, and these may be considered to belong to the iridovirus core genes. The *Rana gryliovirus* (RGV) ORF 53R, which is a homolog of the putative core gene 118L, has been shown to encode a novel iridovirus envelope protein (Zhao et al., 2008). The CIV proteome shares thirteen viral protein homologs with Singapore grouper iridovirus (SGIV) virion proteins identified by two independent mass spectrometric approaches (Chen et al., 2008; Song et al., 2004).

Previous phylogenetic studies on ascoviruses were based on comparative analyses of the capsid protein, DNA polymerase, thymidine kinase, and ATPase III, and led to the hypothesis that ascoviruses may have evolved from invertebrate iridoviruses (Stasiak et al., 2003). The proteomic analysis of CIV performed here showed that 16 ORFs encoding CIV virion proteins have homologs in one or more ascoviruses (Asgari et al., 2007; Bideshi et al., 2006; Bigot et al., 2008; Stasiak et al., 2000; Wang et al., 2006). Nine CIV structural proteins have homologs in *Heliothis virescens* ascovirus 3e (HvAV3e), thirteen have homologs in *Trichoplusia ni* ascovirus 2c (TnAV2c), eleven in *Spodoptera frugiperda* ascovirus (SfAV1a) and six in *Diadromus pulchellus* ascovirus 4a (DpAV4a). The gene products of six of the eleven SfAV1a homologs were also found in the proteome of SfAV1a virions (Tan et al., 2009a). A homolog of the SfAV1a virion protein P64, which was recently shown to be a major DNA binding protein with proposed DNA condensing activity (Tan et al., 2009b) is not encoded in the CIV genome.

Three of the identified CIV virion ORFs are found in one or more ascoviruses, but not in other iridoviruses (209T, 422L and 374R). One of these (422L) is the only CIV virion ORF with a baculovirus homolog (*Cydia pomonella* granulovirus ORF34; genus *Betabaculovirus*). ORF 337L has homology to an entomopoxvirus gene (Table 1, Fig. 2). These results underscore the evolutionary distance of iridoviruses from both baculoviruses and entomopoxviruses and the closer relation to ascoviruses. Despite the proposed close evolutionary relation between the symbiotic

Table 2
Structural proteins of CIV identified by LC-MS/MS with 1 peptide. The ORFs are ordered by the mass of the encoded proteins (column 3).

ORF	NCBI Accession No	Molecular mass (kDa)	Peptide sequence	Protein coverage (% by amino acids)	MH+	Delta m/z (ppm)	z	Xcorr
317L	AAK82178	43.95	IVNLIPQGQFQAK	3.11	1455.832	−0.30	2	1.77
130R	AAB94451	23.18	ICFSEQPLLDDFSNK	7.46	1812.847	1.04	2	2.86
307L	AAK82168	22.86	LKPLGYLNSLQ	5.58	1245.720	0.33	2	1.81
395R	AAK82255	17.28	YAINNENQYR	6.62	1284.597	−0.72	2	2.54
010R	AAK81948	12.84	TGSMVCSSTR	8.33	1085.471	3.19	2	2.34
342R	AAK82203	9.33	IQAQNYATMGIYN-QGSQIR*	21.59	2156.055	2.74	2	3.73
227L	AAK82088	7.72	TFAYEVPIR[a]	14.30	1095.583	1.49	2	2.61
104L	AAB94434	7.05	RVACSPR*	12.30	845.441	2.01	2	2.78

[a] The same peptide was measured multiple times in different gel slices.

ascovirus DpAV4a and *Chilo* iridescent virus (Bigot et al., 2009) the number of CIV virion proteins with homologs in DpAv4a is limited in comparison to the other ascoviruses.

Although the morphology of the virions of members of the family *Ascoviridae* differs considerably from that of viruses of the family *Iridoviridae*, evidence is mounting that the ascoviruses and iridoviruses shared a common ancestor. Phylogenetic analyses based on proteins found in most enveloped dsDNA viruses provide strong evidence that ascoviruses evolved from iridoviruses, despite the marked differences in the characteristics of the virions belonging to these two families and differences in their cytopathology (Bigot et al., 2008). The conservation of structural proteins between CIV and ascoviruses further supports the hypothesis of common ancestry.

In conclusion, this is the first detailed study towards the determination of the virion proteins of an invertebrate iridovirus. This study will contribute to a better understanding of the molecular mechanisms underlying CIV virion assembly, CIV entry into cells, the initial steps of early iridovirus gene expression and the cell to cell movement of this virus.

Materials and methods

Preparation of virus particles and gel electrophoresis

CIV was propagated in larvae of the wax moth, *Galleria mellonella*, isolated as described by Marina et al. (1999) and further purified by 25–65% sucrose density gradient centrifugation. The purified CIV particles were checked for quality by transmission electron microscopy and quantified by UV spectroscopy. The purified particles were denatured and the proteins were separated by 12% one-dimensional SDS-PAGE. The gel was stained with colloidal blue and the gel lane containing the virion proteins was cut into six segments based on a comparison with molecular markers. Each gel piece was sliced and dehydrated with acetonitrile (100%) (ACN). After vacuum drying, the gel segments were incubated in 10 mM dithiothreitol in 50 mM ammonium bicarbonate (ABC buffer) at 57 °C for 1 h and subsequently in 55 mM iodoacetamide (Sigma) in ABC buffer at room temperature for 1 h. After a final wash step with ABC buffer the gel material was dried.

Trypsin digestion and LC-MS/MS

In-gel protein digestions were performed using sequencing grade modified porcine trypsin (Promega, Madison, WI) in ABC buffer at 37 °C for 15 h, after which the digests were centrifuged at 6000 g. The supernatants were collected, and the remaining gel pieces were extracted with 5% triflouroacetic acid (TFA) and then with 15% ACN /1% TFA. The extracts were combined with the supernatants of the original digests, vacuum-dried, and the dried material was dissolved in 20 µl 0.1% formic acid in water. The peptides resulting from this digestion were analyzed by LC-MS/MS. To this aim, 18 µl of the samples were concentrated over a 0.10×32 mm Prontosil 300-5-C18H (Bischoff, Germany) pre-

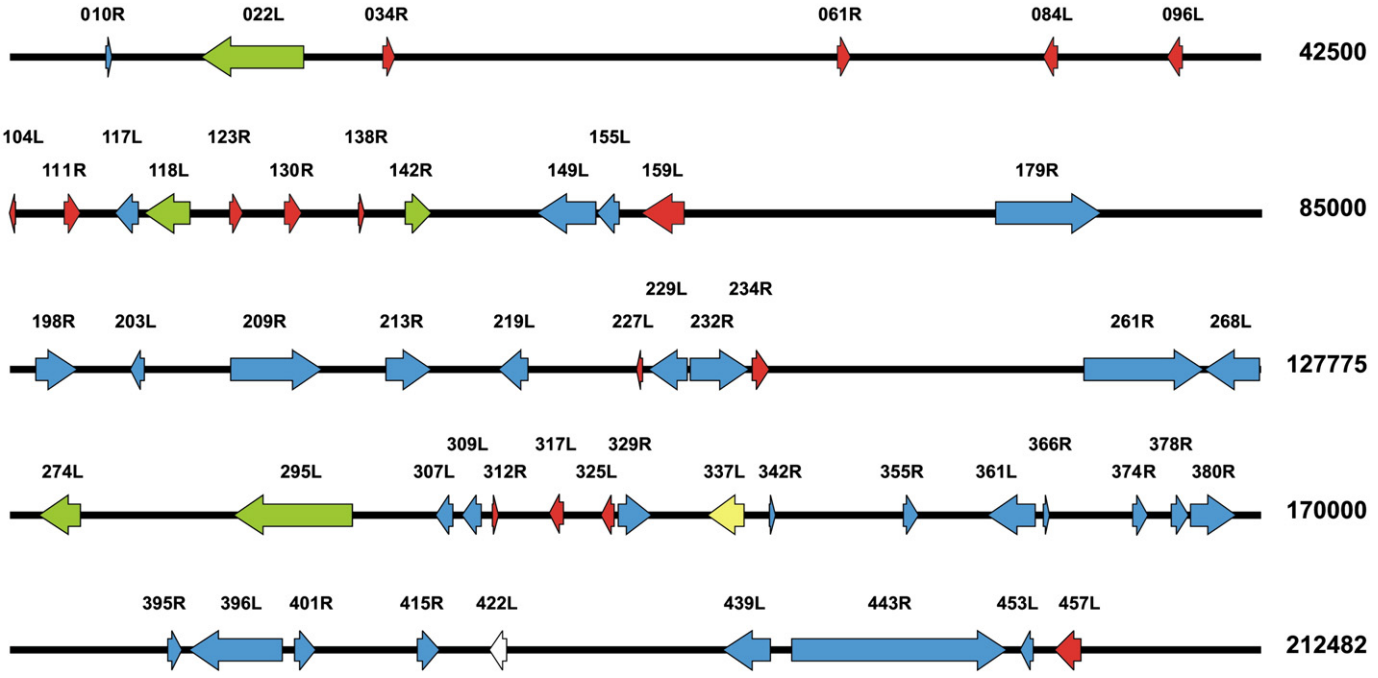

Fig. 2. Linearized genomic presentation of the 54 CIV structural protein ORFs determined by LC-MS/MS. Arrows indicate the positions and the direction of gene transcription (R or L). Red arrows are ORFs unique to CIV, green arrows represent ORFs present in all sequenced iridovirus genomes. The yellow and the white ORFs, have an entomopox- and baculovirus homolog, respectively. The remaining ORFs are indicated in blue. Genomic positions are indicated on the right in base pair number.

Table 3
List of CIV virion proteins identified by LC-MS/MS ordered by mass with homolog in other iridoviruses and/or ascoviruses.*

Invertebrate		Vertebrate											Ascoviridae‡
Irido-virus	Chloririd-virus	Ranavirus						Lymphocystivirus		Megalocytivirus			
CIV	IIV3	ATV	TFV	FV3	SGIV	GIV	STIV	LCDV-C	LCDV-1	ISKNV	RBIV	OSGIV	
443R	91L												
295L	**16R**	**72R**	**45R**	**41R**	**57L**	**29L**	**45R**	**234R**	**92R**	**76L**	**72L**	**75L**	**[a]144R, [b]43R, [c]84L**
179R	35R	60R	29R	27R	78L	44R	31R	172R	110R				[a]129L, [b]58R, [c]90R
022L	**87L**	**7L**	**9L**	**9L**	**60R**	**30L**	**11L**	**75L**	**70L**	**63L**	**59L**	**63L**	**[a]15R, [b]161R, [c]9R**
261R	91L												
209R													[a]76R, [b]115L, [c]64R
396L	91L, 8L												
268L	74L												
149L	113L												
232R	84L	84L					21R						[b]141R
439L	35R								110R	114L		111L	[c]90R
	38R												
	98L												
361L	24R							223L	23R				[a]101L, [b]102R, [c]114R
380R	10L	84L	19R	19R	39L	17L	21R	13L, 45R, 149R, 165L	5L, 42R, 47R, 50R,				
	11L				150L+	83L		174R,184R, 200L	51R, 88L				
213R	51L												
118L	**6R**	**53L**	**55R**	**53R**	**88L**	**49L**	**55R**	**157R**	**35L**	**7L**	**8L**	**8L**	**[b]157L, [d]5L**
198R	69L												
274L	**14L**	**14L**	**96R**	**90R**	**72R**	**39R**	**96R**	**43L**	**80L**	**6L**	**7L**	**7L**	**[a]55R, [b]153R, [c]41R,[d]19R**
229L	46R	3R	4R	229L	16L	2L	5R						
337L	47R	1L	2L	2L	19R	4R	2L	38R	89L		85L		[b]129L, [c]54R
329R	99R												
219L	36R, 91L												
142R	**101R**	**25R**	**85L**	**80L**	**84L**	**46L**	**87L**	**186R**	**74R**	**87R**	**83R**	**85R**	**[a]26R, [b]8R, [c]22R, [d]18L**
155L	113L												
401R	68R												
117L	107R	83L	20R	20R	038L	16L	23R	73R	109R				
415R	18L												
309L	63R												
422L													[d]8R, [d]9R, [d]14L
307L	33L	11R	100L	94L	98R	56R		152L	9R	86R			[a]142R, [c]86L, [d]4R
378R	100L	84L	19R	19R	39L	17L	21R	13L	50R				[b]141R
355R	104L	67R	40R	37R	61R	31L	41R	147L	43L	5L	6L		[a]108R, [b]93L, [c]109L
374R													[b]1R
203L	85L												
395R	1R												
453L	41R												
366R		63R	33R	32R			35R						
010R	43R												
342R	115R												

*ORFs in bold are conserved in all analyzed iridio- and ascovirus genomes.
‡The a-d indices for the ascovirus ORFs refer to the following species: [a] HvAV3e, *Heliothis virescens ascovirus 3e* (Asgari et al., 2007), [b] TnAV2c, *Trichoplusia ni ascovirus 2c* (Wang et al., 2006), [c] SfAV1a, *Spodoptera frugiperda ascovirus 1a* (Bideshi et al., 2006), [d] DpAV4a, *Diadromus pulchellus ascovirus 4a* (Bigot et al., 2008; Stasiak et al., 2000). The names of the other viruses are abbreviated as follows; CIV, *Chilo iridescent virus* (Jakob et al., 2001); IV3, *Aedes taeniorhynchus iridescent virus* (Delhon et al., 2006); ATV, *Ambystoma tigrinum stebbensi virus* (Jancovich et al., 2003); TFV, *Tiger frog virus* (He et al., 2002); FV3. *Frog virus 3* (Tan et al., 2004); SGIV, *Singapore grouper iridovirus* (Song et al., 2004); GIV, *Grouper iridovirus* (Tsai et al., 2005); STIV, Soft-shelled turtle iridovirus (Huang et al., 2009); LCDV-C, *Lymphocystis disease virus* - isolate China (Zhang et al., 2004); LCDV-1, *Lymphocystis disease virus 1* (Tidona and Darai, 1997); ISKNV, *Infectious spleen and kidney necrosis virus* (He et al., 2001); RBIV, *Rock bream iridovirus* (Do et al., 2004); OSGIV, *Orange-spotted grouper iridovirus* (Lü et al., 2005).

concentration column at a flow of 6 µl/min for 5 min. Peptides were eluted from the pre-concentration column and loaded onto a 0.10×200 mm Prontosil 300-3-C18H analytical column with a gradient of 10% to 35% ACN in 0.1% formic acid at a flow of 0.5 µl/min for 50 min. After that the percentage of ACN was increased to 80% (with 0.1% formic acid) in 3 min as a column cleaning step. Between the pre-concentration and analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P875 Upchurch microT. Full scan positive mode Fourier transform mass spectra (FTMS) were measured between mass-to-charge ratios of 380 and 1400 with a LTQ-Orbitrap spectrometer (Thermo electron, San Jose, CA, USA). MS/MS scans of the four most abundant doubly and triply charged peaks in the FTMS scan were recorded in a data dependent mode in the linear trap (MS/MS threshold = 10.000). All MS/MS spectra obtained with each run were analyzed with Biowork 3.1.1 software (Thermo Fisher Scientific, Inc.). A maximum of 1 allowed differential modification per peptide was set for oxidation of methionines and de-amidation of asparagine and glutamine residues. Carboxamidomethylation of cysteines was set as a fixed modification. Trypsin specificity was set to fully enzymatic and a maximum of 3 missed cleavages with monoisotopic precursor and fragment ions. The mass tolerance for peptide precursor ions was set to 10 parts per million (10 ppm = 0.01 op m/z 1000 amu) and for MS/MS fragment ions to 0.5 Da. An Invertebrate iridescent virus 6 protein database was used for the analysis (AF303741; created July 31, 2001; downloaded from www.ncbi.nlm.nih.gov/sites/entrez) after adding a list of commonly observed contaminants like: BSA (P02769, bovine serum albumin precursor), trypsin (P00760, bovine), trypsin (P00761, porcin), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human) and keratin K1CI (P35527, human). A decoy database was created by adding the reversed sequences using the program SequenceReverser from the MaxQuant package (Cox and Mann, 2008), resulting in a total of 1058 proteins in the database. To identify the proteins in the CIV virions, the MS/MS spectra obtained from the LC-MS/MS were searched against the CIV ORF database using Bioworks 3.3.1 (Table 1). The peptide identifications obtained were filtered in Bioworks with the following filter criteria: ΔCn>0.08, Xcorr>1.5 for

charge state 2+, Xcorr>3.3 for charge state 3+ and Xcorr>3.5 for charge state 4+ (Peng et al., 2003). Only those proteins that showed a Bioworks Score factor (Sf) larger then 0.6 were considered.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.05.038.

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