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Genes encoding cytoplasmic intermediate filament proteins of vertebrates revisited: Identification of a cytoplasmic intermediate filament protein in the sea anemone *Nematostella*

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ABSTRACT

The cytoskeleton is crucial in determining cell architecture, division, motility, transport processes and in local control of signal transduction. Relatives of actin and tubulin are expressed in all phyla, underlining the fundamental importance of conserved cytoskeletal functions. Intermediate filament proteins have evolved in parallel with tissue diversity in the animal kingdom, likely from the demand to adapt one class of cytoskeletal proteins to cell type-restricted functions. Up to now, the evolutionary origin of cytoplasmic intermediate filament proteins remains unknown. Using a known gene encoding a cytoplasmic intermediate filament protein from the hemichordate *Saccoglossus kowalevskii*, we have identified the first corresponding gene in the sea anemone *Nematostella*, tentatively named cytovec. Our data reveal a relationship of cytovec with *Hydra vulgaris* nematocilins A and B that also lack a CAAX box. In light of additional recent findings, our data show that cytoplasmic intermediate filament genes are present in the common ancestor of Cnidaria and Bilateria.

Introduction

The cytoskeleton is crucial in determining cell architecture, division, motility transport processes and in local control of signal transduction. Whereas relatives of actin and tubulin are expressed in all phyla intermediate filament proteins have evolved in parallel with tissue diversity in the animal kingdom, likely from the demand to adapt one class of cytoskeletal proteins to cell type-restricted functions (Fuchs and Weber, 1994; Wickstead and Gull, 2011). The multigene family of metazoan intermediate filament proteins covers two groups: the nuclear lamins and the cytoplasmic intermediate filament (IF) proteins. Their sequence homologies are particularly obvious in the protostomic IF proteins, because the central coiled coil domains have the same length as the lamins, while in deuterostomic species the central domain of IF proteins is shortened (Dodemont et al., 1990; Doring and Stick, 1990).

A major difference between lamins and IF proteins concerns two features of the lamins: a nuclear localization signal, usually 4 consecutive basic residues in the carboxy-terminal tail domain, and the C-terminal isoprenylation site C-a-a M, which in a stepwise reaction gives rise to chargeless carboxy terminal cysteine (Gruenbaum et al., 2005).

RNA interference studies under standard conditions showed that at least 5 of the IF proteins of the nematode *C. elegans* are essential in the earliest stages of embryonic development (Karabinos et al., 2001). The family of mammalian cytoplasmic IF proteins is much larger (Hesse et al., 2001) and its expression patterns has been reviewed (Iwatsuki and Suda, 2010; Windoffer et al., 2011). In accordance with the large number of mammalian IF genes, at least 25 human IF genes show mutations which give rise or contribute to diseases (Bonifas et al., 1991; Vassar et al., 1991; Szeverenyi et al., 2008; http://www.interfil.org/index.php). While type III proteins like desmin form in vivo and in vitro homopolymeric IF, the epithelial keratins are obligatory heteropolymers based on dimers containing a type I and a type II keratin. The type IV neurofilament proteins can have larger molecular weights due to unique extensions of the C-terminal tail domains.

Metazoa cover the more basal phyla of the radiata, which have radial symmetry and the large group of bilateral animals, which have bilateral symmetry (Wehner and Gehring, 2007). When genomic information became available for the first representative of the radiata, we screened the developing genome of the sea anemone *Nematostella vectensis* for representatives of lamin and IF genes. Although we found a lamin gene, we and others failed to detect IF genes (Zimek and Weber, 2008). This was unexpected,

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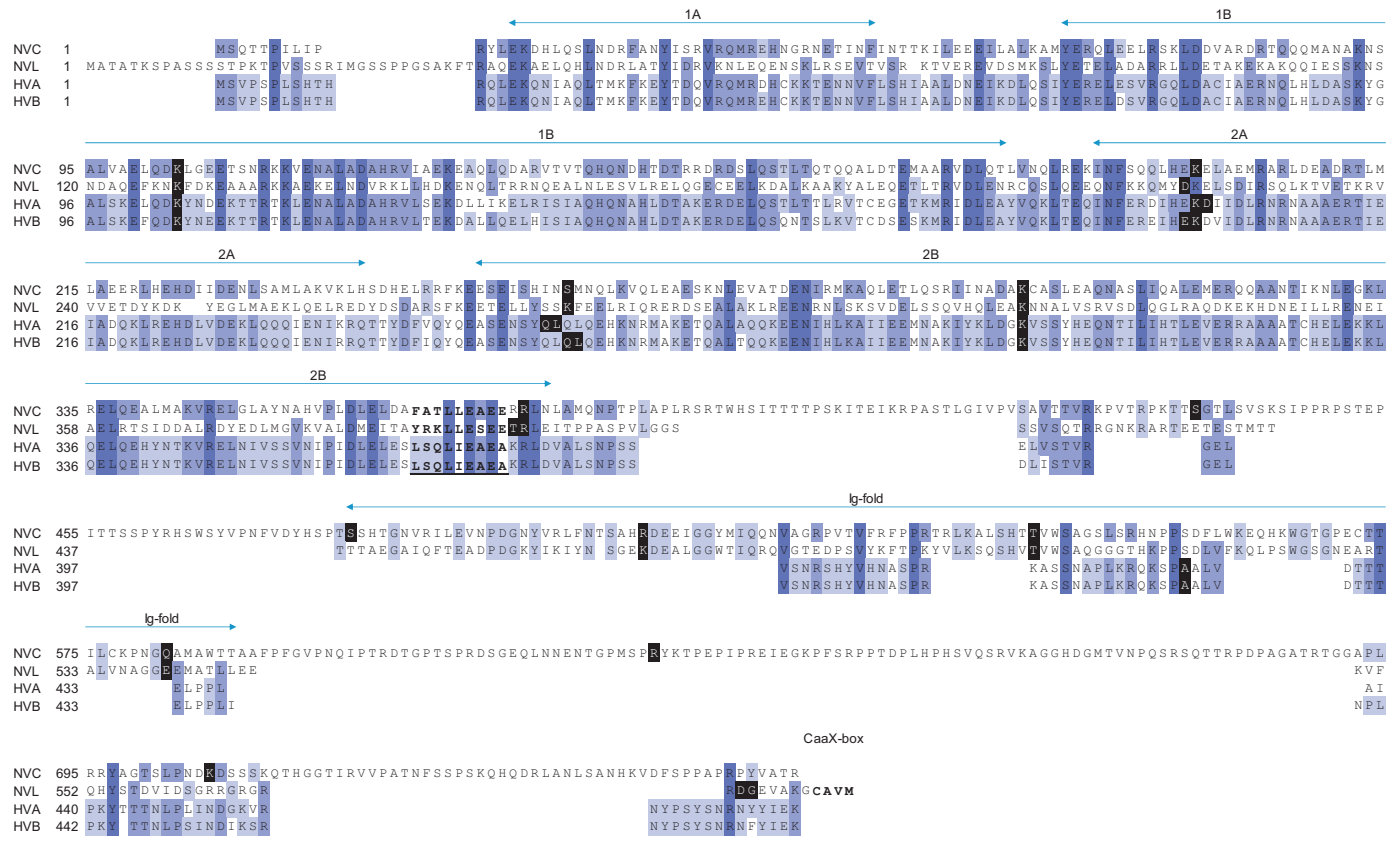
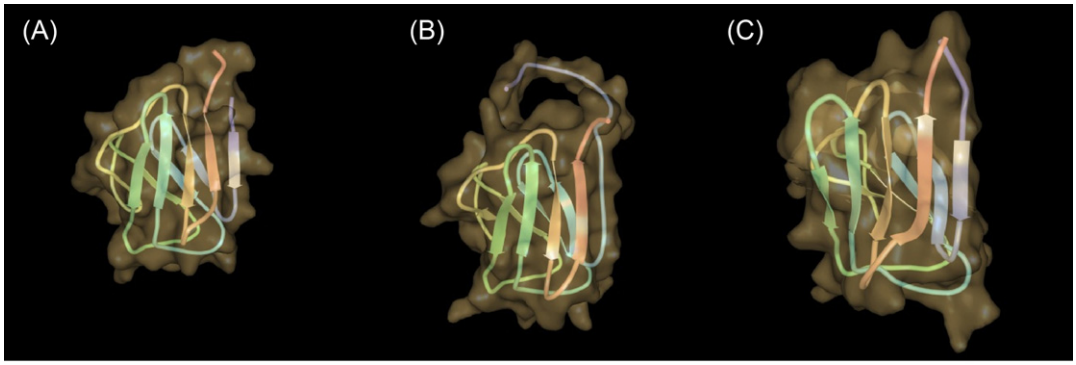


NVC Nematostellavectensiscytovec
NVL Nematostellavectensis lamin
HVA Hydra vulgaris nematocilin A (BAG48261.1)
HVB Hydra vulgaris nematocilin B (BAG48262.1)

Fig. 1. Alignment of the predicted cytoplasmic IF protein cytovec from the cnidarian *Nematostella vectensis* (upper line) with the previously published predicted lamin sequence from the same species and with *Hydra v.* nematocilin A and B. Intron positions are labeled black on white background in all figures to mark them very clearly. Marking of 2 amino acids indicate intron position between indicated positions without codon interruption. Marking of 1 amino acid indicates that the exon–intron boundary interrupts the corresponding codon. Rod domains are marked and denoted 1A, 1B, 2A and 2B. The YRKLLEGEE consensus at the end of coil 2B is underlined in black. Ig-fold denotes the immunoglobulin fold domain and CaaX-box the C-terminal isoprenylation site. Identical amino acids among all proteins are marked with a dark blue line. Light blue denotes identical amino acids among 2 or more related sequences. NVC *Nematostella vectensis* cytovec, NVL, *Nematostella vectensis* lamin (XM_001629238), HVA *Hydra vulgaris* nematocilin A (BAG48261.1), HVB, *Hydra vulgaris* nematocilin B (BAG48262.1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

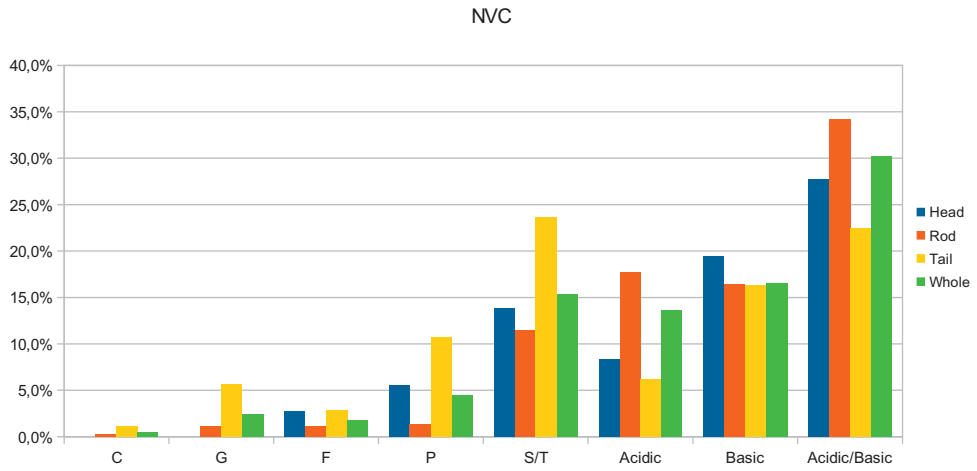


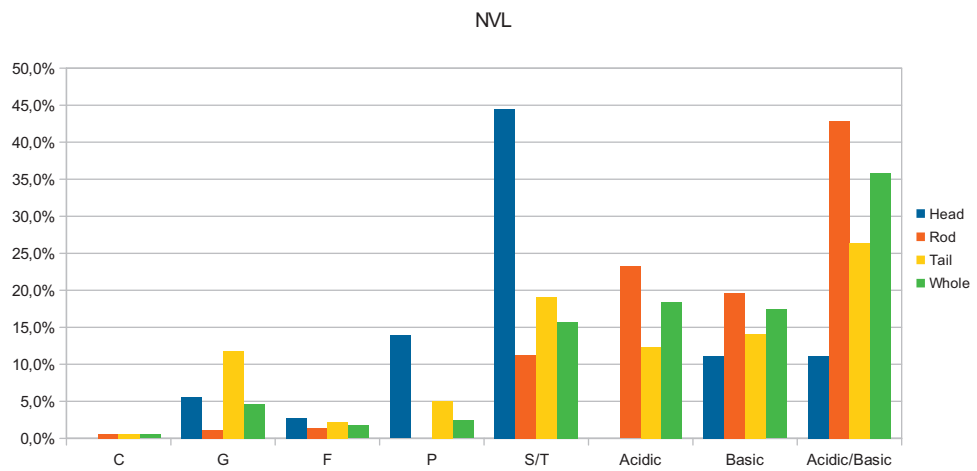
(A) Homo sapiens lamin A/C
(B) Nematostellavectensiscytovec
(C) Nematostellavectensislamin

Fig. 2. Structure of Homo sapiens lamin A/C Ig fold and predicted structure of corresponding domain in *Nematostella vectensis* cytovec and lamin.

Table 1
Distribution of selected amino acid residues among head, rod and tail domains of *Nematostella vectensis* cytovec (NVL) and lamin (NVL). Note the enrichment of Gly residues in the tail of lamin and the enrichment of Pro in the tail of cytovec. Also, Ser/Thr are prevalent in the head domain of the lamin. Top part lists distribution of the amino acids cysteine (C), glycin (G), phenylalanine (F), prolin (P), serin and threonine (S/T), of acidic and basic and their ratio along the head, rod and tail domains of *Nematostella* cytovec (NVC) and lamin. Lower part, graphical display of amino acid distribution along head, rod, tail and entire protein.

	NVC				NVL			
	Head (%)	Rod (%)	Tail (%)	Whole (%)	Head (%)	Rod (%)	Tail (%)	Whole (%)
C	0.0	0.3	1.1	0.5	0.0	0.5	0.6	0.5
G	0.0	1.1	5.6	2.4	5.6	1.1	11.8	4.7
F	2.8	1.1	2.8	1.7	2.8	1.4	2.2	1.7
P	5.6	1.4	10.7	4.5	13.9	0.0	5.1	2.4
S/T	13.9	11.5	23.6	15.3	44.4	11.2	19.1	15.7
Acidic	8.3	17.8	6.2	13.6	0.0	23.2	12.4	18.4
Basic	19.4	16.4	16.3	16.6	11.1	19.7	14.0	17.4
Acidic/basic	27.8	34.2	22.5	30.2	11.1	42.9	26.4	35.9

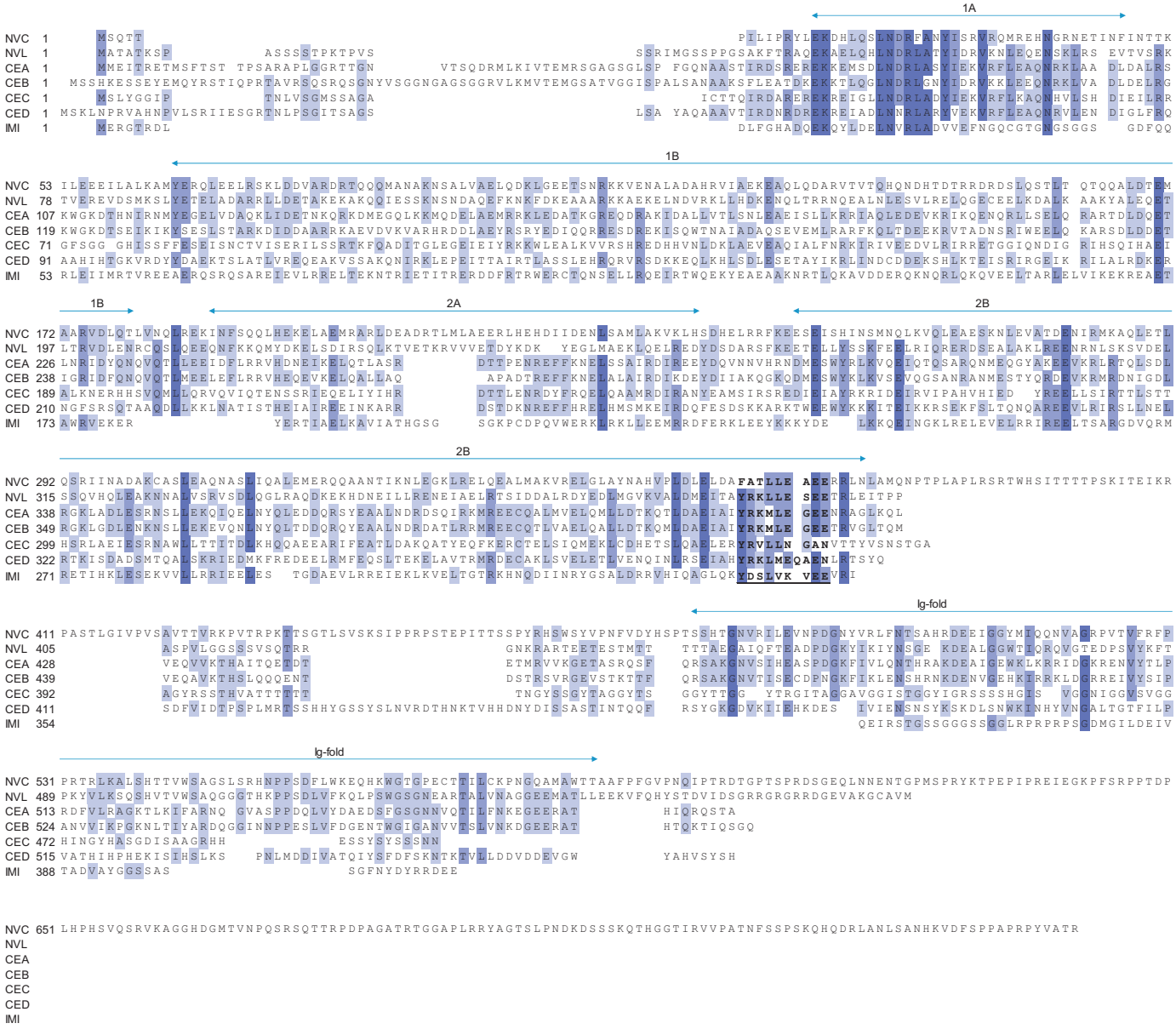




since previous electron microscopical studies documented a wealth of cytoplasmic IF in the radiata (see for instance Bartnik and Weber (1989)). Here we repeated the screen using however as probe a gene from metazoa closer related to radiata than the mammalian genes, which were earlier used. In our work, we identified the cytoplasmic protein cytovec, a putative IF-forming protein with the highest degree of similarity to the cytosolic IF protein from *Clytia hemispherica* and from nematocilin A and B from *Hydra vulgaris* (Hwang et al., 2008).

Materials and methods

We analyzed the genome assembly of the sea anemone (*N. vectensis*). The *Nematostella* genome assembly 1.0 was produced by the Joint Genome Institute (JGI). It has a 7.8-fold coverage in 10,804 scaffolds with a total sequence length of 356 Mbp of sequence. Roughly half of the genome is contained in 181 scaffolds all at least 473 kb in length. Graphics were created with the open source suite LibreOffice, available at http://www.documentfoundation.org. To compare the *N. vectensis* cytovec with other metazoans IF, we used the following protein sequences: *Acropora digitifera* (adi_v1.13506), *C. hemispherica* (CL437Contig1), *Caenorhabditis elegans*: Intermediate filament protein ifa-1 (P991), ifb-1 (Q19289), ifc-1 (O45168), ifd-1 (Q86DC6), lamin (Q21443), *Ciona intestinalis* cIF (XP_002120583.1), nuclear lamin (NP_001093902.1), *Danio rerio* lamin A (AAI63799.1), *Drosophila melanogaster* lamin C (NP_523742.2), *Homo sapiens* desmin (NP_001918.3), keratin type I cytoskeletal 18 (NP_954657.1), keratin type II cytoskeletal 8 isoform



NVC	Nematostellavectensiscytovec
NVL	Nematostellavectensis lamin
CEA	Caenorhabditis elegans Intermediate filament protein ifa-1 (P991)
CEB	Caenorhabditis elegans Intermediate filament protein ifb-1 (Q19289)
CEC	Caenorhabditis elegans Intermediate filament protein ifc-1 (O45168)
CED	Caenorhabditis elegans Intermediate filament protein ifd-1 (Q86DC6)
IMI	Isotomurusmaculatusisomin (ACJ66847.1)

Fig. 3. Alignment of the predicted cytoplasmic IF protein cytovec from the cnidarian *Nematostella vectensis* (upper line), the previously published predicted lamin sequence from the same species, with *C. elegans* IF proteins IFA-1, IFB-1, IFC-1 and IFD-1 and with isomin from *Isotomurus maculatus*. NVC, *Nematostella vectensis* cytovec, NVL, *Nematostella vectensis* lamin (XM_001629238), CEA, *Caenorhabditis elegans* intermediate filament protein ifa-1 (P991), CEB, *Caenorhabditis elegans* intermediate filament protein ifb-1 (Q19289), CEC, *Caenorhabditis elegans* intermediate filament protein ifc-1 (O45168), CED, *Caenorhabditis elegans* intermediate filament protein ifd-1 (Q86DC6), IMI, *Isotomurus maculatus* isomin (ACJ66847.1).

2 (NP_002264.1), lamin A/C transcript variant 1 (AAW32540.1), neurofilament light polypeptide (NP_006149.2), vimentin (NP_003371.2), *H. vulgaris* nematocilin A (BAG48261.1) nematocilin B (BAG48262.1), *Isotomurus maculatus* isomin (ACJ66847.1), *N. vectensis* lamin (XM_001629238), *Saccoglossus kowalevskii* cIF (XP_002736176.1), lamin A/C-like (XP_002734960.1), *Strongylocentrotus purpuratus* cIF (XP_796075.2), nuclear intermediate filament protein (NP_999665.1), *Xenopus* (*Silurana*) *tropicalis* lamin A/C (NP_001039148.1). These sequences were either manually selected or determined by the NCBI BLASTP search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For the BLASTP search we used the *N. vectensis* cytovec sequence.

The sequence alignments were calculated by using the multiple alignment tool MUSCLE v3.8.31 (http://www.drive5.com/muscle/). For further visualizing of these alignments we used the Jalview v 2.7 tool (http://www.jalview.org/).

Protein structures were predicted by the phyre 2 tool (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index)

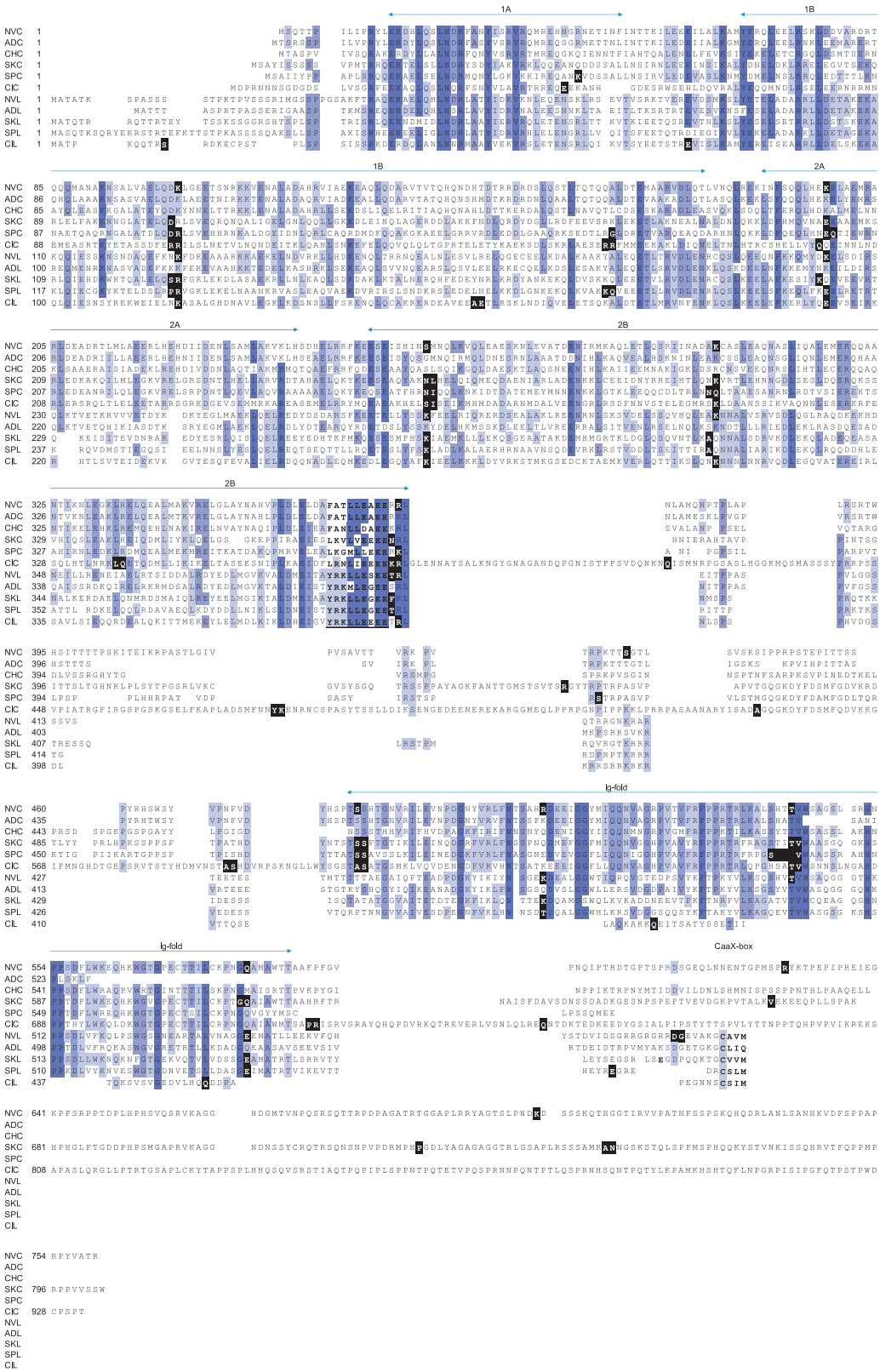
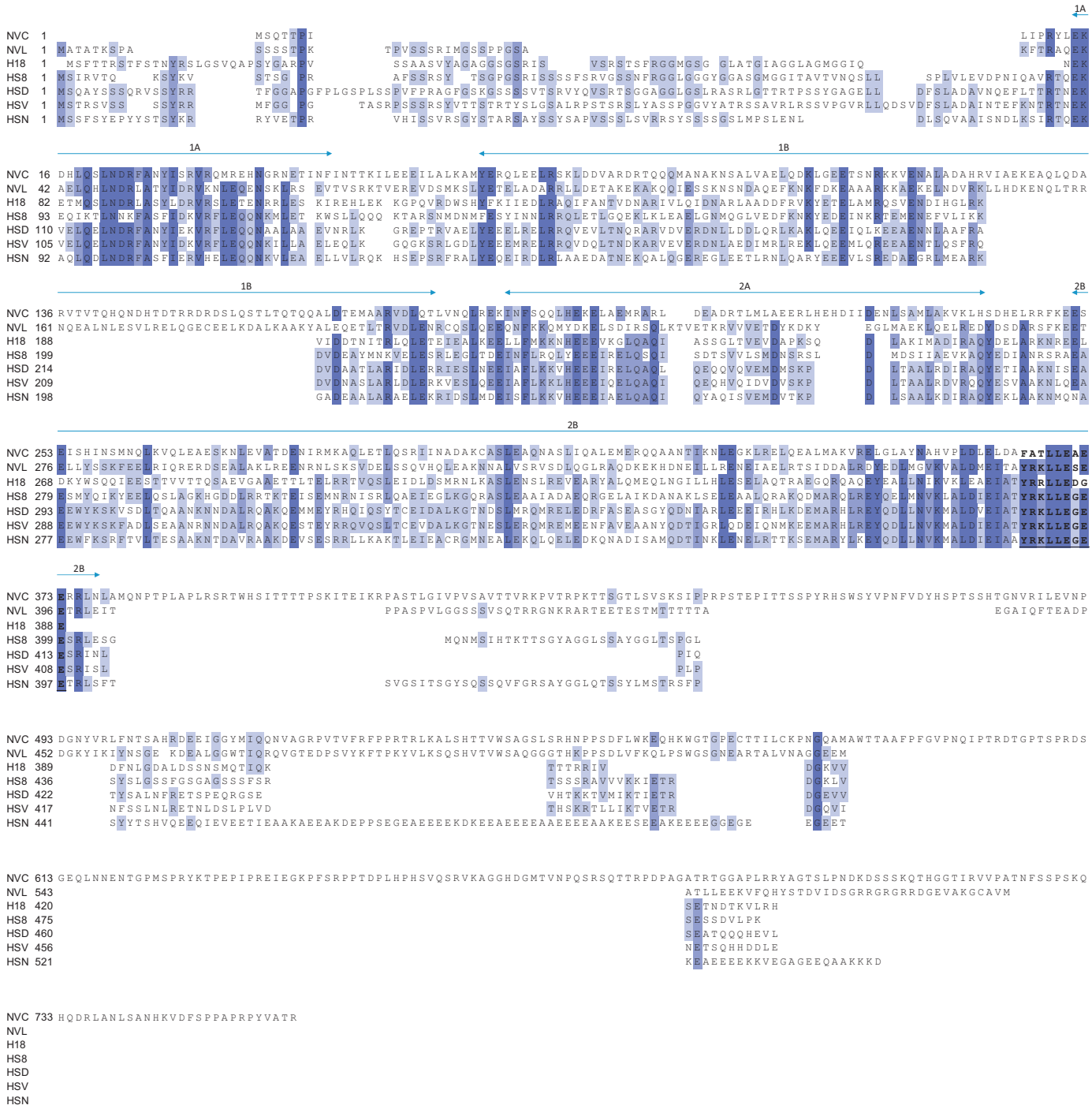
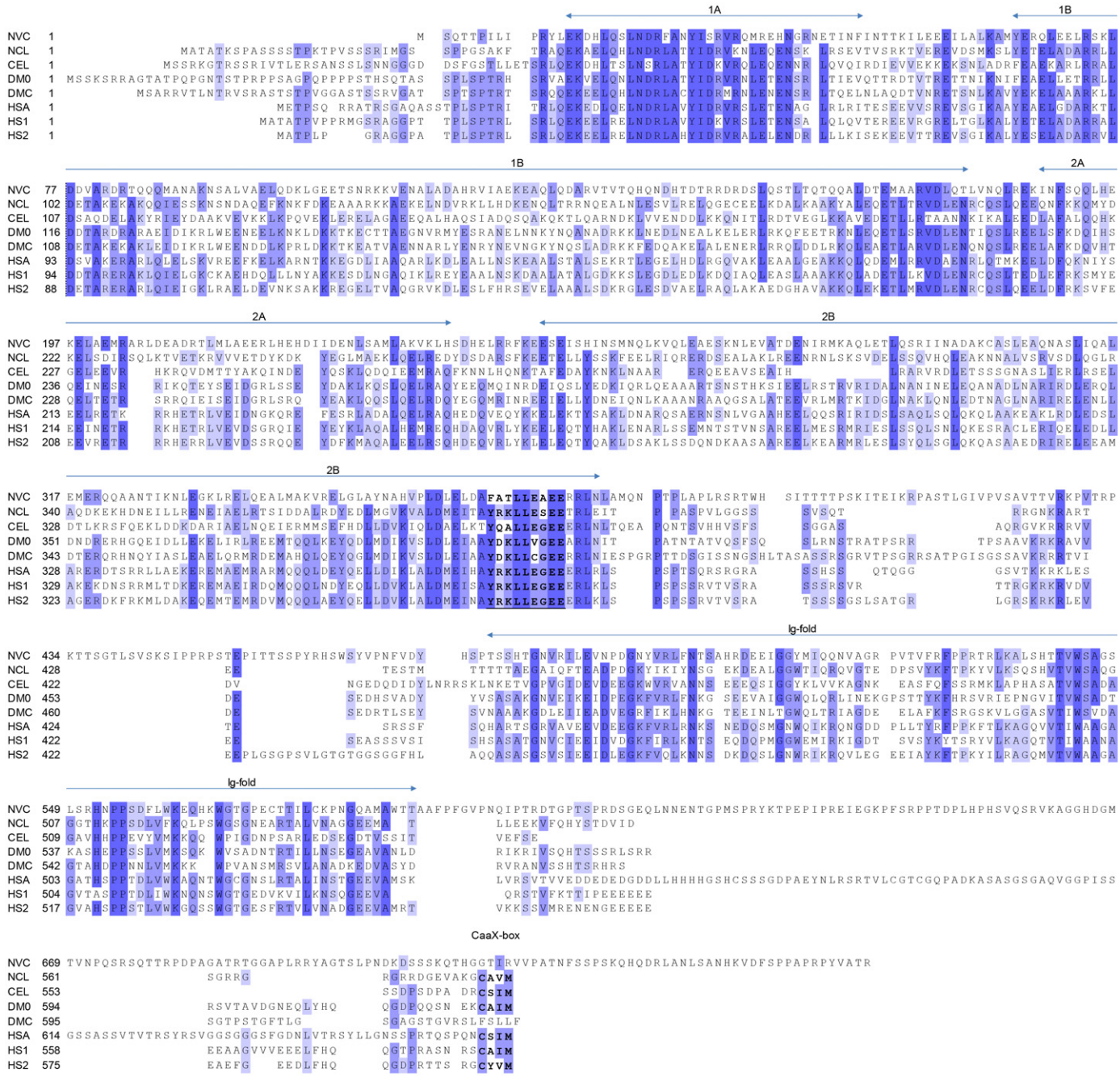


Fig. 4. Alignment of the predicted cytoplasmic IF protein protein cytovec from the cnidarian *Nematostella vectensis* (upper line) with predicted cytoplasmic IF proteins from ADC, *Acropora digitifera** (adi_v1.13506), CHC, *Clytia hemispherica*. (CL437Contig1), SKC, *Saccoglossus kowalevskii* (XP_002736176.1), SPC, *Strongylocentrotus purpuratus* (XP_796075.2), CIC, *Ciona intestinalis* (XP_002120583.1), NVL, *Nematostella vectensis* lamin, ADL, *Acropora digitifera* lamin* (adi_v1.19659), SKL, *Saccoglossus kowalevskii* lamin-A/C-like (XP_002734960.1), SPL, *Strongylocentrotus purpuratus* nuclear intermediate filament protein (NP_999665.1), CIL, *Ciona intestinalis* nuclear lamin (NP_001093902.1). *For *Clytia hemispherica* and *Acropora digitifera*, only incomplete transcriptome data are available, precluding to mark exon overlaps.



NVC Nematostellavectensiscytovec
NVL Nematostellavectensis lamin
H18 Homo sapiens keratin type I cytoskeletal 18 (NP_954657.1)
HS8 Homo sapiens keratin type II cytoskeletal 8 isoform 2 (NP_002264.1)
HSD Homo sapiens desmin (NP_001918.3)
HSV Homo sapiens vimentin (NP_003371.2)
HSN Homo sapiens neurofilament light polypeptide (NP_006149.2)

Fig. 5. Alignment of the predicted cytoplasmic IF protein cytovec from the cnidarian *Nematostella vectensis* (upper line) with the previously published predicted lamin sequence from the same species and with vertebrate cytoplasmic IF proteins. H18, *Homo sapiens* keratin type I cytoskeletal 18 (NP_954657.1), HS8, *Homo sapiens* keratin type II cytoskeletal 8 isoform 2 (NP_002264.1), HSD, *Homo sapiens* desmin (NP_001918.3), HSV, *Homo sapiens* vimentin (NP_003371.2), HSN, *Homo sapiens* neurofilament light polypeptide (NP_006149.2). Note that sequence conservation is restricted to start of coil 1A and the end of coil 2B.



NVC Nematostellavectensiscytovec
NCL Nematostellavectensis lamin
CEL Caenorhabditis elegans lamin (Q21443)
DM0 Drosophila melanogaster lamin Dm0 (P08928)
DMC Drosophila melanogaster lamin C (NP_523742.2)
HSA Homo sapiens lamin A/C transcript variant 1 (AAW32540.1)
HS1 Homo sapiens lamin B1 (AAC37575.1)
HS2 Homo sapiens lamin B2 (AAH06551.1)

Fig. 6. The previously published predicted lamin sequence from the same species and with lamins from various species. Note the presence of a CaaX motif in all except *Drosophila mel*. lamin C. CEL, *Caenorhabditis elegans* lamin (Q21443), DML, *Drosophila melanogaster* lamin C (NP_523742.2), DRL, *Danio rerio* lamin A (AAI63799.1), XTL, *Xenopus tropicalis* lamin A/C (NP_001039148.1), HSL, *Homo sapiens* lamin A/C transcript variant 1 (AAW32540.1).

using the intensive modeling Method. Visualized are the IG-folds by RCSB PDB Protein Workshop 3.9.

For the reconstructing of phylogenetic tree we used the maximum likelihood approach, which is available in SeaView v 4.3.0 by using the PhyML package v 3.0.1 (http://pbil.univ-lyon1.fr/software/seaview.html). The calculated tree was stored in the svg format and afterword modified in Inkscape (http://inkscape.org) for a better visualization.

Results

Identification of a cytoplasmic nematostella IF protein

Using the previously described IF gene from the hemichordate *S. kowalevskii* (Zimek and Weber, 2002) as a probe on the genome information of *Nematostella*, we located a potential IF gene on scaffold 94 (nt. 50739–65390). The deduced protein sequence is shown in Fig. 1, which also gives the position of the 12 introns from the 5′-end. Intron sizes are 800 bp, 285 bp, 930 bp, 380 bp, 1554 bp, 110 bp, 5716 bp, 322 bp, 697 bp, 211 bp, 957 bp and 435 bp. Position of introns 1, 2, 3, 4, 5, 8, 9 and 10 are conserved with those in *Nematostella* lamin (Zimek and Weber, 2008). Cytovec shares 19.7% identical amino acids with the previously described lamin gene of *Nematostella*, and ~23.5% with the cytoplasmic nematocilins of *H. vulgaris* (Fig. 1). The highest degree of sequence identity is found at the rod end domains and in the middle of coil 1B. The IF consensus sequence YRKLLEGEE at the rod end is well conserved except that the Tyr residue is replaced by a Phe in cytovec. The *Nematostella* cytovec gene is clearly not a second lamin gene, since it lacks the nuclear localization signal and the C-a-a-M motive indicated for the lamin genes. However, it contains a recognizable immunoglobulin-like domain although structure prediction algorithms predict an arrangement distinctly different from that of Nematostella lamin (Fig. 2). Whereas the nuclear localization signal in bilateral metazoa involves 4 consecutive basic residues, the lamins from the two species of the radiata currently known show only 3 basic residues in the sequences KRSR (*Hydra*) and KRAR (*Nematostella*) (Zimek and Weber, 2011). The deduced protein sequence of 760 amino acids corresponds to a size of 85.4 kDa and shows a length like in protostomic metazoa (Zimek and Weber, 2008). The sizeable number of Nematostella EST sequences covering the entire open reading frame support its expression. Table 1 lists the amino acid distribution in the two known *Nematostella* proteins. Across the rod domain, *Nematostella* lamin has a much higher ratio of acidic/basic (42.9%) residues compared to cytovec (34.2%). Another distinguishing feature is the prevalence of Ser/Thr residues in the lamin head domain. The overall amino acid distribution differs strongly from that of human keratins (Strnad et al., 2012).

Relationship of nematostella cytovec to IF proteins from other species

Fig. 3 reveals a distinct relationship between the 2 *Nematostella* IF proteins, the recently described isomin (*I. maculatus*; Mencarelli et al., 2011) and cytoplasmic IF proteins from *C. elegans*. The relationship among the *Nematostella* IF sequences (~19.6%) is closer than to *C. elegans* proteins (~9.9–14% identity) with well conserved rod motifs at the start of coil 1A and the end of coil 2B. Isomin is very distantly related to cytovec and shares ~7.7% identical amino acids. To further examine the relationship between cytovec, cytoplasmic and nuclear IF proteins, additional comparisons were performed. Figs. 4–6 provide data on cytovec's relationship with selected IF sequences. The limited EST sequences from the anthozoan

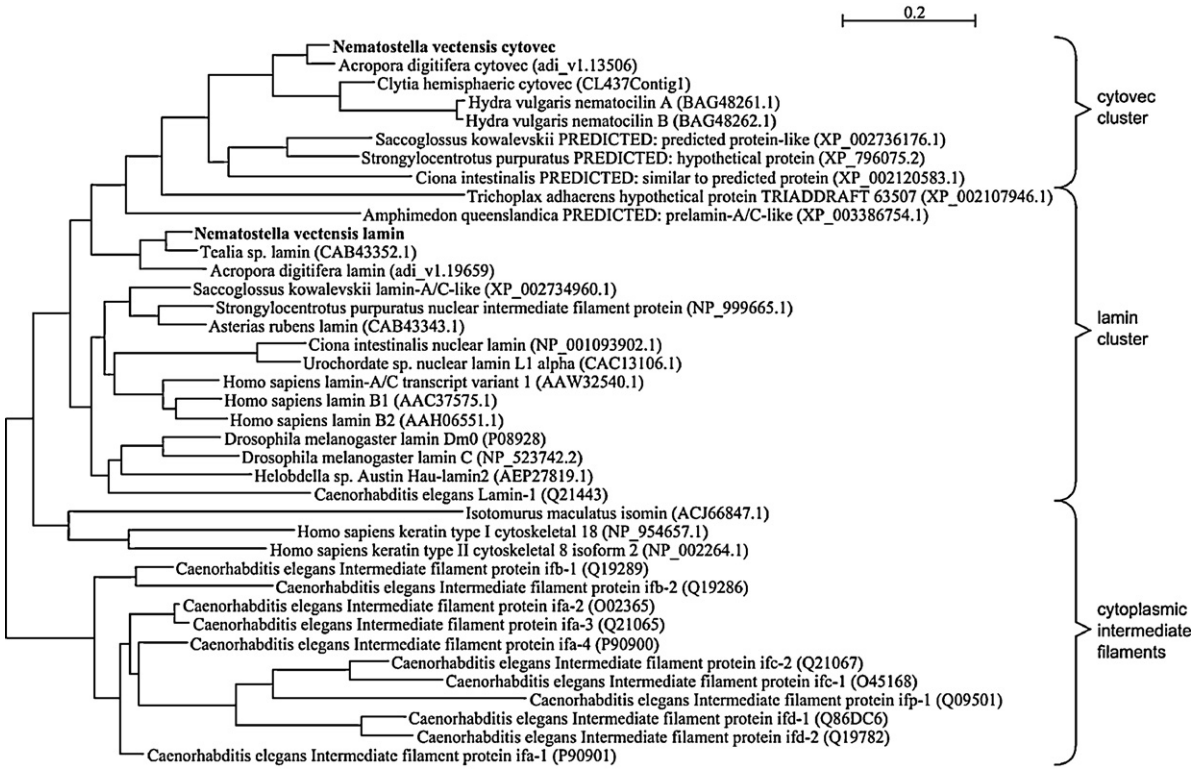


Fig. 7. Distance analysis of *Nematostella vectensis* and other IF proteins. Reconstruction of the phylogenetic tree was performed using SeaView with the PhyML 3.0 software package. GenBank accession numbers are indicated in brackets. Note the isolated position of isomin. Three related groups encompassing relatives to Nematostella vect. Cytovec ("cytovec cluster"), to Nematostella vect. Lamin ("lamin cluster") and cytoplasmic IF proteins ("other intermediate filament proteins"), in addition to the isolated isomin, are recognized.

A. digitifera and the hydrozoan *Clytia hemispherica* reveal 44.8% sequence identity between the entire *Clytia* protein and the first 627 amino acids of cytovec (Fig. 4). The C-terminal extension of 135 residues in the latter have no counterpart in the Acropora and Clytia sequences. With regard to other IF proteins, the extent of sequence identity is low (between 9% and 11.8%). The rod end domains, in particular the end of coil 2B, are well conserved, supporting the classification of cytovec as cytoplasmic IF protein. Because of the closeness of these values it is currently impossible to decide which mammalian IF protein is the obvious counterpart of *Nematostella* IF cytovec. Attempts to get information via flanking genes as previously done for lamin genes (Zimek and Weber, 2011) were not possible because of the gaps in the genome of *Nematostella*. We note however that in the primitive bilateral animal, the nematode *C. elegans*, there is firm in vivo and in vitro evidence for a keratin-like obligatory heteropolymer system, but no indication for an additional homopolymer system (Karabinos et al., 2003). The phylogenetic tree analysis (Fig. 7) supports a relatively close relationship between cytovec, the cytoplasmic IF proteins of the anthozoan *A. digitifera*, the hydrozoans *Clytia hemispherica*, *H. vulgaris*, *S. purpuratus*, *S. kowalevskii* and *C. intestinalis*. It further confirms the grouping of Nematostella lamin with other lamins. These two groups are distantly related from other cytoplasmic IF proteins and from isomin.

Discussion

Among cytoskeletal proteins, intermediate filament proteins represent the most recent acquisition in evolutionary terms, coinciding with the occurrence of multicellularity (Wickstead and Gull, 2011). Using as probe a gene from metazoa closer related to radiata than the mammalian genes, we identified the putative cytoplasmic protein cytovec in *Nematostella*, which reveals that cytoplasmic intermediate filament genes are present in the common ancestor of Cnidaria and Bilateria. Sequence comparisons showed that this protein is related to the bona fide IF proteins nematocilin A and B of *H.* vulgaris and to the cytoplasmic IF proteins of the anthozoan *A. digitifera* and the hydrozoans *C. hemispherica*. The organization of the nematocilins in a central filament of the cnidocil of *H. vulgaris* where they are surrounded by microtubules suggest an architectural role of IF early in evolution (Hwang et al., 2008). Whether cytovec forms typical IF remains to be analyzed. Possibly, expression of a cytovec cDNA in *E. coli* may help to address its filament-forming capacity. The proteins could be purified by standard conditions and their assembly properties studied. We suggest that a further screen for cytoplasmic IF genes should be made when the genome of *Nematostella* is completed. The closest metazoan phyla for which abundant cytoplasmic intermediate filament proteins have been documented by electron microscopy are the Cnidarians and Ctenophora (Bartnik and Weber, 1989). It will be interesting to see whether evolution of cytoplasmic IF proteins coincides or follows metazoan evolution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejcb.2012.08.003.

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