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EF-hand proteins in onychophorans as compared to tardigrades and other ecdysozoans

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ABSTRACT

To learn more about the regulation of muscle contraction in the structural and functionally different muscles in Ecdysozoa, we have analysed the four EF-hand proteins calmodulin (CaM), troponin C (TnC), essential myosin light chain (eMLC) and regulatory myosin light chain (rMLC) in the velvet worm Peripatoides *sp. These proteins are typified by a helix-loop-helix motif and trigger the actin–myosin interaction in muscle tissue and non-muscle cells by coordinative binding of calcium ions. CaM (632 bp) and TnC (853 bp) revealed 4 and 2 potential calcium-binding domains, respectively. Based on analysis of both MLCs, only the rMLC (798 bp) showed one potential N-terminal EF-hand domain and a consensus phosphorylation sequence motif, which suggests a phosphorylation of this myosin light chain. We have compared these results with previously published data from the eutardigrade* Hypsibius klebelsbergi *and found striking conformity between both taxa. Further, we compared the new sequences with corresponding sequences from nematodes and arthropods retrieved from GenBank.*

Key words: EF-hand proteins, onychophorans, tardigrades, Ecdysozoa, phylogeny.

INTRODUCTION

The EF-hand protein superfamily comprises Ca^{2+}-binding proteins that are characterised by a helix-loop-helix motif containing amino acid residues which coordinatively bind calcium ions by their oxygen-containing side chains or alpha-carbonyl oxygens. Essential positions within the loop and the beginning of the second helix were marked 1[X], 3[Y], 5[Z], 7[-Y], 9[-X] and 12[-Z] (Bhattacharya *et al*., 2004). Functional Ca^{2+}-binding in the narrow physiological Ca^{2+}-concentration range from 10^{-7} to 10^{-5} M is the trigger for actin-myosin interaction (Niggli, 1999) leading to the active or resting state of muscle cells. The EF-hand proteins troponin C (TnC), essential myosin light chain (eMLC) and regulatory myosin light chain (rMLC), which are linked to actin and/or myosin, and calmodulin (CaM) confer the Ca^{2+} signal and can regulate the *on* and *off* state of actin myosin interaction (Szent-Györgyi, 1975; Lewit-Bentley and Rety, 2000). Regulation of muscle contraction is actin- and/or myosin-linked and if both control systems are fully functional in a single muscle cell, it is referred to as dual regulated (Lehman and Szent-Györgyi, 1975). The actin-associated regulatory proteins troponin (Tn) and tropomyosin (Tm) can block myosin binding sites on actin at low cellular calcium. Calcium-binding to the Tn subunit TnC leads to considerable structural changes and unblocks actin (Gordon *et al*., 2000). Myosin-linked control is constituted by its associated myosin light chains (MLCs). Direct binding of Ca^{2+} or a calmodulin (CaM) mediated phosphorylation of the myosin light chains can switch *on* the myosin and allow its interaction with actin (Mooseker and Cheney, 1995).

In the body wall muscle of annelids, myosin is apparently regulated by the reversible binding of Ca^{2+} to the rMLC, whereas in molluscan muscle, Ca^{2+} is bound by the eMLC (Carlhoff and D'Haese, 1988; Szent-Györgyi *et al*., 1999; Himmel *et al*., 2009). However, in muscles of nematodes and arthropods studied so far the phosphorylation of the rMLC is essential for myosin activation. Therefore the type of regulation of muscle contraction in onychophorans and tardigrades, which both are said to play a key role for understanding arthropod evolution, may be of special interest.

Recently we have determined and analysed the complete cDNA sequences of proteins of the EF-hand superfamily (CaM, TnC and both MLCs) in the eutardigrade *Hypsibius klebelsbergi* Mihelčič, 1959 (Prasath *et al*., 2012). Our findings suggest also a dual regulated system with a calcium-dependent tropomyosin-troponin complex bound to the actin filaments and a phosphorylation of the rMLC switching *on* and *off* both actin and myosin.

In the present study we determined the nucleotide and amino acid sequences of the EF-hand proteins of the velvet worm *Peripatoides* sp. and compared these sequences with corresponding sequences of the eutardigrade *H. klebelsbergi* and several other Ecdysozoa. With these studies we expect further information how muscle contraction in onychophorans that possess muscles with sarcomere-like arrangement of the myofilaments similarly organised as in tardigrades (Lanzavecchia and Camatini,

1979) is regulated. Furthermore, we examined whether EF-hand proteins can be used as phylogenetic characters, when applied within a distinct clade such as Ecdysozoa that have structurally and functionally different muscles, but nevertheless have the same regulatory mechanism.

METHODS

Adults of *Peripatoides* sp. were commercially purchased (www.exotic-pets.co.uk). Before dissection, animals were cold-anaesthetised on ice for 30 min. Internal organs and intestine were removed and only the body wall with legs were used for RNA isolation. Total RNA was purified from body wall with legs of adult animals using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNAs were generated using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA).

Conserved EF-hand amino acid regions from arthropods were used as a basis to design degenerated primer pairs for all four EF-hand proteins. Using this set of degenerated primers (Supplementary Tab. 1) we could initially amplify short (200-300 bp) cDNA fragments. The full cDNA sequences were obtained by 5'- and 3'-rapid amplification of cDNA ends (RACE) according to the manual of the SMART RACE cDNA Amplification Kit (Clontech) followed by cloning and sequencing the RACE products. We used premixed PCR master mixes (Peqlab, Erlangen, Germany) for the initial amplification of short cDNA fragments using 100 ng cDNA and 0.5 µM of each primer with a total sample volume of 25 µL. Amplification was run with 5 min initial denaturation at 94°C followed by 35 cycles of 30 sec denaturation at 94°C, 1 min annealing at 50-55°C and 3 min elongation at 72°C. The program ended with a final incubation step for 10 min at 72°C. PCR products were separated in 1.2% agarose gels in TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, 1 µg/mL ethidiumbromide) and custom sequenced by Seqlab (Göttingen, Germany). The resulting PCR fragments were cloned into pJET 1.2/blunt vector using CloneJET PCR Cloning kit (Fermentas, Burlington, Canada) and transformed into TOP F10 cells (Invitrogen). Individual colonies were picked as templates for colony PCR reactions employing the supplemented primers pJET-forward (5'CGACTCACTATAGGGAGAGCGGC-3') and pJET-reverse (5'-AAGAACATCGATTTTC-CATGGCAG-3'). Products within the expected size range were sequenced and verified by BLAST-search analysis and comparison with expressed sequence tags (ESTs) of several ecdysozoans available in GenBank. These verified sequence fragments were used for RACE-PCRs to obtain the complete sequences.

Sequence analysis

All protein sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and the UniProt database (www.uniprot.org).

For phylogenetic analysis, the web service platform ATGC: PhyML was used (http://www.atgc-montpellier.fr/phyml/, Guindon *et al*., 2010).

Sequences were aligned using MUSCLE (Edgar, 2004). ProTest (Abascal *et al*., 2005) was used to determine that the Le and Gascuel (LG) algorithm was the optimum amino acid replacement model for accurate phylogeny resolution (Le and Gascuel, 2008).

Trees were estimated with PhyML using maximum likelihood (Guindon and Gascuel, 2003) and visualised with TreeDyn (Chevenet *et al*., 2006). The generated tree was finally edited with Figtree v1.22 (http://tree.bio.ed.ac.uk/software/figtree/). Percentages of 1000 bootstrap resamplings are given. Only bootstrap values above 50% are shown.

RESULTS

As a start we obtained short (200-300 bp) cDNA fragments for all four EF-hand proteins by using degenerated primers. These verified sequence fragments were used for RACE-PCRs to obtain the complete sequences. Sequences obtained have been deposited in GenBank (Accession numbers: Calmodulin, KC603609; Troponin C, KC603610; regulatory myosin light chain, KC603611; essential myosin light chain, KC603612).

We assume we have identified the major isoforms for all four EF-hand proteins expressed in the velvet worm *Peripatoides* sp. All four EF-hand proteins show the same set of canonical EF-domains also identified in the eutardigrade *H. klebelsbergi* (Tab. 1). Analyses of canonical domains were performed according to Marsden *et al*. (1990) and Gifford *et al*. (2007).

Calmodulin

The cDNA obtained for CaM was 632 nucleotides in length with an open reading frame (ORF) of 450 bp (150 amino acids) and a calculated molecular weight of 16785.6 Da. As typical for CaM, it is a highly acidic protein (pI of 4.10) due to a high content of aspartate and glutamate residues (24.8%). It lacks tryptophan and cysteine. The CaM of *Peripatoides* sp. contains four canonical helix-loop-helix EF-hand domains. All four Ca^{2+}-binding loops always show aspartate at both coordinates X and Y, whilst glutamate is found at coordinate -Z. Furthermore, coordinates Z and -X are occupied by essential oxygen-containing side chain residues (Fig. 1). Sequence comparison with CaM of *H. klebelsbergi* shows 96% amino acid and 86% nucleic acid identity (Tab. 1; accession numbers: G9B6R4 and HM628689, respectively).

Troponin C

The cDNA of TnC shows a length of 853 bp encoding

a protein with an ORF of 477 bp (159 amino acids). It has a calculated molecular weight of 17970.2 Da. The deduced amino acid sequence shows an excess of aspartate (10.1%) and glutamic acid (14.6%) which results in a pI of 4.22. N- and C-terminal region contain each two EF-hand domains. EF-hand I differs from the canonical pattern at coordinate Z and –Z occupied by glutamine and methionine and at –X by histidine. EF-hand domain III shows glutamine at coordinate Y, where, however, an aspartate or asparagine is preferred. EF-hand domains II and IV display a canonical pattern from which a specific binding of Ca^{2+} can be deduced (Fig. 2). BLAST search shows the highest score and 76% amino acid and 72% nucleic acid identity with *H. klebelsbergi* (accession number: G9B6R3 and HM628688.1, respectively).

Essential myosin light chain

The eMLC cDNA is 835 bp long and contains an ORF of 471 bp. It encodes an acidic protein of 157 amino acids (pI of 4.82) with a calculated molecular weight of 17670.1 Da. Sequence analysis revealed two C-terminally located non-canonical degenerated EF-hand domains (Fig. 3).

Regulatory myosin light chain

The rMLC cDNA (798 bp) contains an ORF of 495 bp. The full-length translation product is predicted to be an acidic protein of 165 amino acids (pI of 4.81) with a calculated molecular weight of 18737.0 Da. The N-terminal region shows a phosphorylatable motif with threonine-serine at position 16 and 17. Both residues are flanked upstream by a consensus recognition sequence of positively charged amino acids including KKR xxR xxS xVF (position 9-20) essential for the catalytic activity of myosin light chain kinases. Each N- and C-terminus shows a single calcium-binding EF-hand domain. EF-hand I contains a canonical Ca^{2+}-binding loop pattern. EF-hand III shows at Ca^{2+}-binding-loop coordinates Y, Z, -X and -Z amino acid substitutions which lead to the loss of essential, negatively charged residues (Fig. 4). A database search with BLAST reveals the highest score with the rMLC of *H. klebelsbergi* with 72% amino acid and 82% nucleic acid identity (accession numbers: G9B6R2 and HM628687.1, respectively).

DISCUSSION

In a previous article (Prasath *et al*., 2012), we proposed a dual regulation of somatic muscle contraction in *H. klebelsbergi* (Tardigrada) but could not exclude non-muscle isoforms as whole specimens had to be used for RNA isolation. In the case of *Peripatoides* sp. (Onychophora) we could isolate the RNA mainly from the body wall muscle. The high similarities between the onychophoran and tardigrade sequences we regard as a further indication that also the EF-hand proteins in *H. klebelsbergi* are from somatic muscles. It should be noted that both onychophoran and tardigrade muscle cells exhibit a similar structural organisation (Shaw, 1974; Walz, 1974). They do not show an arrangement in helical fibres with oblique-striation as in the body wall muscle of annelids and nematodes. Rather, they have a sarcomere-like arrangement of their myofilaments which allows nearly unlimited increase of myofibres in diameter and volume (Lanzavecchia and Camatini, 1979). This organisation is regarded as an intermediate stage in the evolution of cross-striated muscle (Camatini *et al*., 1979; Lanzavecchia and Camatini, 1979).

Tab. 1. Sequence properties of the EF-hand proteins of the velvet worm *Peripatoides* sp. and the eutardigrade *Hypsibius klebelsbergi*. The percentages of nucleotide and amino acid identities between the four EF-hand proteins calmodulin, troponin C, essential- and regulatory myosin light chain of *Peripatoides* sp. and *H. klebelsbergi* are shown. Isoelectric point and molecular weight were calculated using *ExPASy's* Compute *pI*/Mw programme (http://web.expasy.org/compute_pi). All four EF-hand proteins show the same set of canonical EF-hand domains.

	CaM	TnC	eMLC	rMLC
Peripatoides sp.				
Total length (bp)	632	853	835	798
ORF (bp/as)	450/150	477/159	471/157	495/165
pI/Mw	4.10 16785.6	4.22 17970.23	4.82 17670.17	4.81 18737.07
Canonical EF-hand domains	4 (I-IV)	2 (II and IV)	0	1 (I)
Hypsibius klebelsbergi				
Total length(bp)	600	824	1015	984
ORF (bp/as)	450/150	459/153	498/166	525/175
pI/Mw	4.09 16836.64	4.17 17354.33	4.71 18473.13	4.87 19052.49
Canonical EF-hand domains	4 (I-IV)	2 (II and IV)	0	1 (I)
	86% nt (96% aa)	72% nt (76% aa)	74% nt (68% aa)	81% nt (72% aa)

CaM, calmodulin; TnC, troponin C; eMLC, essential myosin light chain; rMLC, regulatory myosin light chain; bp, base pairs; ORF, open reading frame; pI, Isoelectric point; Mw, molecular weight; nt, nucleotide; aa, amino acid.

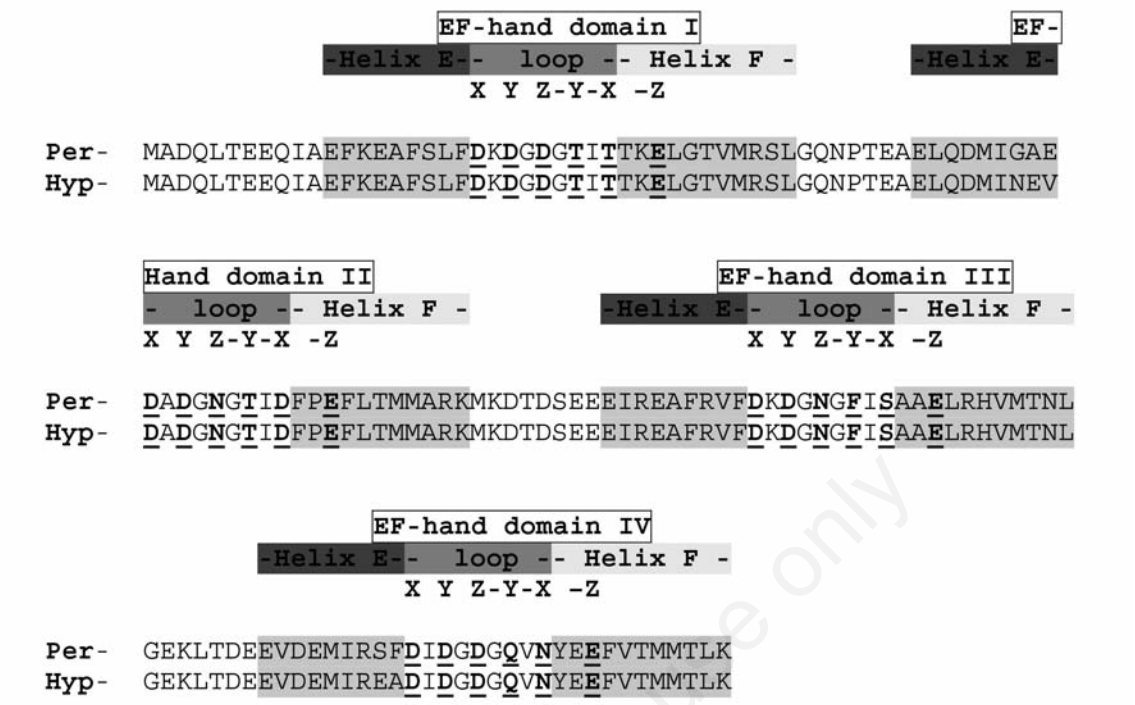

Fig. 1. EF-hand domains and comparison of *Peripatoides* sp. (onychophoran) calmodulin with *Hypsibius klebelsbergi* (eutardigrade). All four EF-hand domains display a canonical Ca^{2+}-binding loop pattern. Calcium coordinating key residues (X, Y, Z, -Y, -X and -Z) which comply with the consensus pattern (for details see text) are bold-underlined, helices shaded. Helices - E, - F and calcium-binding loops are shown as a block above the sequence. The chosen sequence-labeling for the EF-hand domains is likewise used for the troponin C, essential- and regulatory myosin light chain figures. Sequence comparison showed 96% amino acid (86% nucleic acid) similarity.

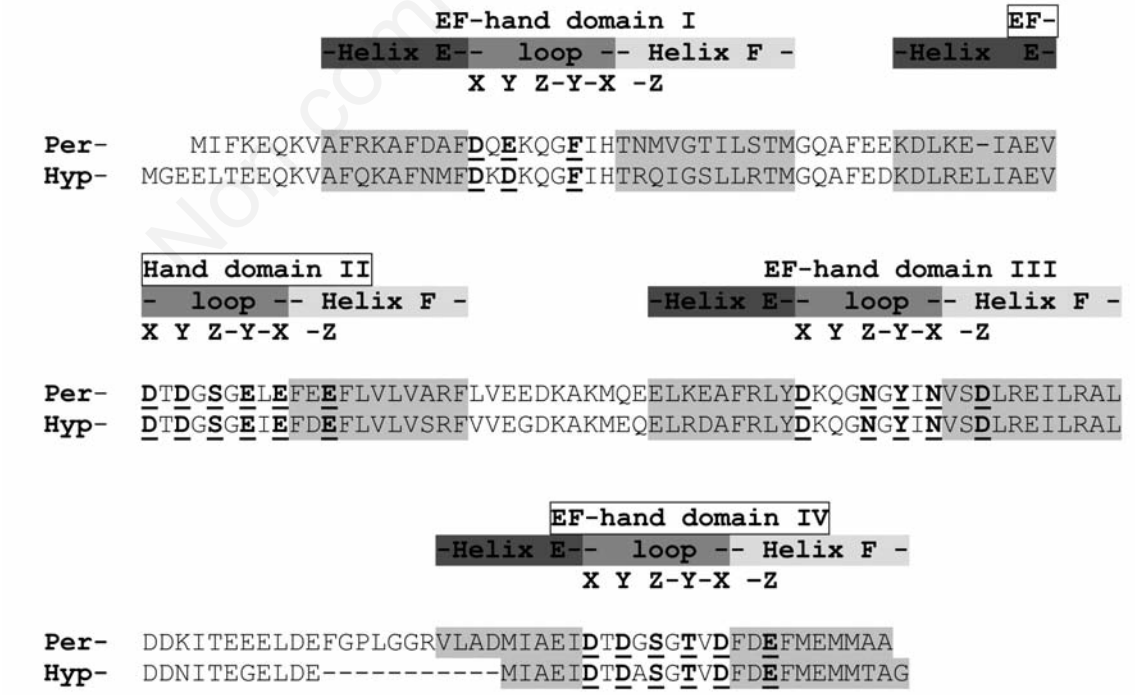

Fig. 2. EF-hand domains and comparison of *Peripatoides* sp. (onychophoran) troponin C with *Hypsibius klebelsbergi* (eutardigrade). The troponin C of *Peripatoides* sp. shows two canonical calcium-binding EF-hand domains, EF-hand domain II and EF-hand domain IV. Sequence comparison showed 76% amino acid (72% nucleic acid) identity. For sequence-labeling see Fig. 1.

As assumed for the tardigrade muscle of *H. klebelsbergi*, the Ca^{2+}-sensitivity of the dual system of muscle regulation in *Peripatoides* sp. is apparently conferred by the actin-linked tropomyosin/troponin complex and direct binding of Ca^{2+} to TnC and by a myosin-linked regulation via the CaM mediated phosphorylation of the rMLC, which both can turn *on* and *off* actin myosin interaction. To our knowledge, the main groups of Ecdysozoa such as nematodes and arthropods apparently use the same kind of muscle regulation. This is noteworthy as the main groups have structurally and functionally different muscles.

Other invertebrates, such as annelids and molluscs also possess a dual regulatory system, but here myosin is regulated by direct calcium-binding to rMLC or to eMLC, respectively (Carlhoff and D'Haese, 1988; Szent-Györgyi *et al*., 1999; Himmel *et al*., 2009).

The EF-hand domain consists of a central loop flanked by two perpendicular helices and can specifically bind calcium ions. The proteins studied here contained in their ancestral form four functional EF-hands, which number is retained only in CaM. The TnCs of invertebrate muscles studied so far reveal the loss of two or even three Ca^{2+}-binding EF-hand domains (Kobayashi *et al*., 1989; Allhouse *et al*., 1999; Ueda *et al*., 2001). In the case of TnC from *Peripatoides* sp. and *H. klebelsbergi* (Prasath *et al*., 2012) the EF-hand domains I and III are degenerated whereas the N-terminal EF-hand II and the C-terminal EF-hand IV are functional in the same way as in arthropods and nematodes studied so far (Kobayashi *et al*., 1989; Allhouse *et al*., 1999; Ueda *et al*., 2001).

Only molluscan eMLCs contain one canonical EF-hand domain (EF-hand III) which has been shown to be capable of Ca^{2+}-binding (Szent-Györgyi *et al*., 1999). In rMLC a single canonical EF hand is present in the N-terminal domain as in all rMLCs we compared. More important for the regulation of onychophoran, tardigrade, nematode, and arthropod muscle cells probably is a phosphorylatable threonine-serine motif in the vicinity of the EF-hand domain flanked by a consensus recognition sequence of positively charged amino acids including KKR xxR xxS xVF, which are essential for the catalytic activity of myosin light chain kinase (Ikebe *et al*., 1994; Gao *et al*., 1995; Kamm and Stull, 2001).

CONCLUSIONS

In our previous article (Prasath *et al*., 2012) we concluded that phylogenetic trees obtained from all sequences available from TnCs, rMLCs, and eMLCs may reflect the evolution of muscle regulatory proteins rather than real

Fig. 3. EF-hand domains and comparison of *Peripatoides* sp. (onychophoran) essential myosin light chain with *Hypsibius klebelsbergi* (eutardigrade). The essential myosin light chain of *Peripatoides* sp. shows two degenerated C-terminal calcium-binding EF-hand domains, EF-hand domain III and EF-hand domain IV. Probable remnants of the ancestral EF-hand domains I and II are indicated. Sequence comparison showed 68% amino acid (74% nucleic acid) identity. For sequence-labeling see Fig. 1.

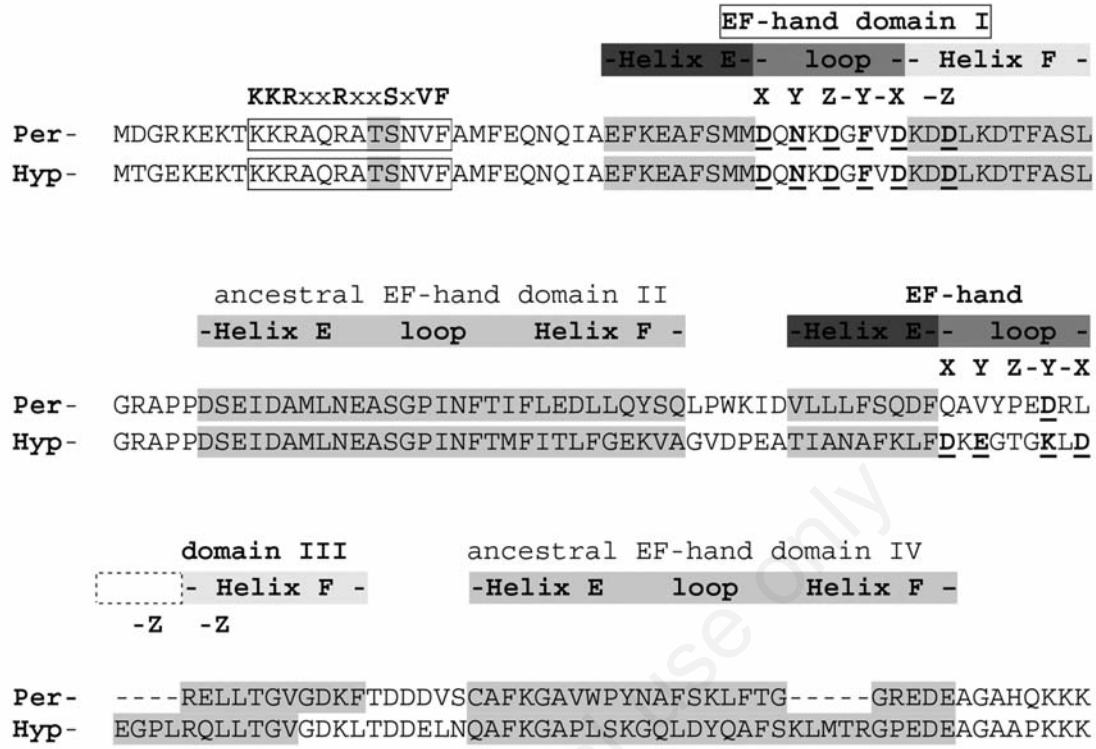

Fig. 4. EF-hand domains and comparison of *Peripatoides* sp. regulatory myosin light chain with *Hypsibius klebelsbergi.* The regulatory myosin light chain of *Peripatoides* sp. shows one canonical calcium-binding EF-hand domain, EF-hand domain I. Residues 1-21 containing the phosphorylatable threonine-serine and the consensus myosin-light-chain-kinase motif (KKRxxRxxSxVF) (framed) are shown. Probable remnants of the ancestral EF-hand domains II and IV are indicated. Sequence comparison showed 72% amino acid (82% nucleic acid) identity. For sequence-labeling see Fig. 1.

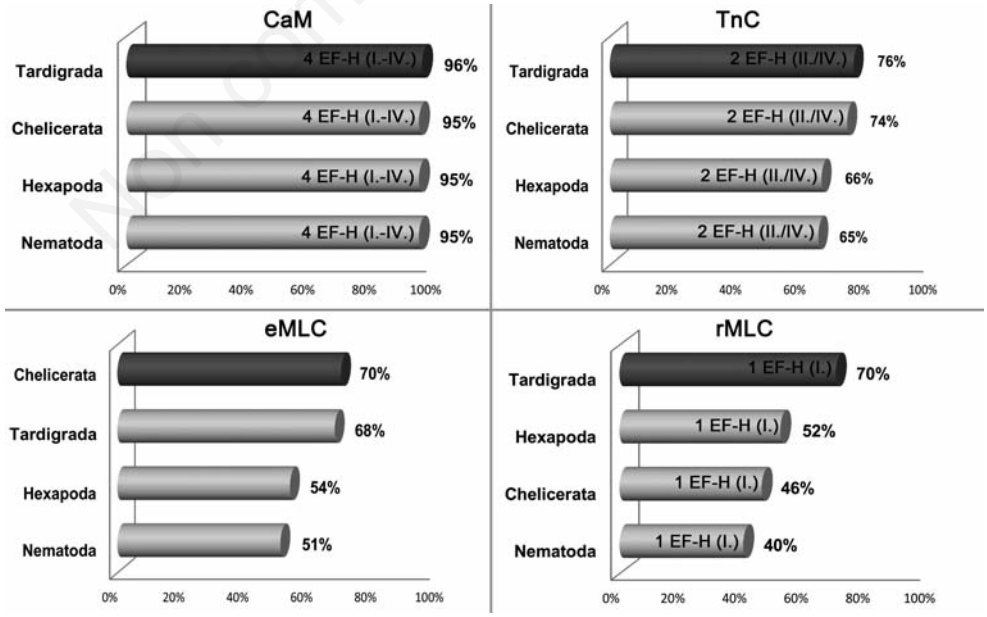

Fig. 5. Comparison of the amino acid sequences of the four EF-hand proteins calmodulin (CaM), troponin C (TnC), essential- (eMLC), and regulatory myosin light (rMLC) chain of the velvet worm *Peripatoides* sp. with the corresponding sequences of Chelicerata, Hexapoda, Nematoda and Tardigrada. Sequence identity to the EF-hand protein of *Peripatoides* sp. is shown as percentage. Numbers of canonical EF-hand domains (EF-H) are given. Within Ecdysozoa the sequences are most similar between *Peripatoides* sp. (Onychophora) and *Hypsibius klebelsbergi* (Tardigrada). Sequences used for comparison are listed in Supplementary Tab. 3.

phylogenetic relationships of the taxa considered (Prasath *et al*., 2012). However, in view of the present results we want to broach this matter again. Nematodes and arthropods as well as onychophorans and tardigrades are included in the Ecdysozoa (Aguinaldo *et al*., 1997; Giribet *et al*., 2001; Regier *et al*., 2005; Mallatt and Giribet, 2006; Roeding *et al*., 2009). As shown herein the deduced amino acid sequences of the examined proteins exhibit a remarkable congruence between *Peripatoides* sp. and *H. klebelsbergi* (Fig. 5). However, for comparison the use of CaM is limited because it is extremely conserved in eukaryotes (Lewit-Bentley and Rety, 2000; Bouche *et al*., 2005). Many studies consider the Panarthropoda, *i.e.* Onychophora +Tardigrada + Arthropoda as monophyletic (Zrzavy *et al*., 1998; Budd 2001; Giribet *et al*., 2000; Regier *et al*., 2005; Telford *et al*., 2008; Rota-Stabelli *et al*., 2010; Campbell *et al*., 2011; Nielsen, 2012; Persson *et al*., 2012; for an association of tardigrades with the Cycloneuralia especially nematodes see Meusemann *et al*., 2010). Some studies place Onychophora basal to the Arthropoda (Giribet *et al*., 2000; Mayer *et al*., 2010), but relationships within Panarthropoda are a matter of debate and all possible combinations have been discussed. Worth noting is the striking similarity of the EF hand proteins in onychophorans with that of Chelicerata (Fig. 5). Previous studies using 12S ribosomal RNA have suggested a sister group relation of Onychophora to this taxon, and onychophorans have even been considered as modified arthropods (Ballard *et al*., 1992). Neuroanatomical traits were interpreted in a similar way (Strausfeld *et al*., 2006). Only the eMLC tree seems to support a close relationship between Onychophora and Tardigrada. However, as our data generally appear to have a limited phylogenetic significance (Supplementary Tab. 2, Supplementary Fig. 1), and as we focused mainly on functional aspects, we do not deepen this discussion herein.

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