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LETTERS TO THE EDITOR

A Mitochondrial Intron in a Verongid Sponge

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Abstract We discovered for the first time a mitochondrial intron in a non-tetillid demosponge, which sheds new light on the interpretation of mitochondrial intron evolution among non-bilaterian animals and has consequences for phylogenetic and DNA barcoding studies. The newly discovered class 1 intron of *Aplysinella rhax* (Verongida) CO1 has an ORF for a putative LAGLIDADG-type and resembles other sponge and cnidarian mitochondrial introns. Our analysis of the *Aplysinella rhax* intron underlines that the patchy distribution of introns in sponges is caused by a combination of horizontal and vertical transmission. Further implications for CO1 phylogenetic and barcoding projects are discussed.

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Introduction

A decade since the publication of the first complete mitochondrial (mt) poriferan genomes (Lavrov et al. 2005), our understanding of animal mtDNA evolution is still incomplete. These genomes revealed major structural differences to their bilaterian counterparts. Subsequently, published mt-genomes of placozoans (Dellaporta et al. 2006), ctenophores (Pett et al. 2011), and calcareous sponges (Lavrov et al. 2013) displayed even more dramatic differences to the relatively homogeneous bilaterian mt-genomes. Several differences can directly be attributed to ancestral traits, successively lost in Bilateria. Additionally, there are several mt-*intragenic* elements only present in sponges and other non-bilaterian mtDNA, such as *repetitive hairpin elements* (see e.g., Erpenbeck et al. 2009). Likewise *mitochondrial introns*, frequently self-splicing and mobile elements, are known from choanoflagellates, ichthyosporeans, and other close relatives of Metazoa (Burger et al. 2003; Burt and Koufopanou 2004), furthermore in Porifera, Cnidaria, and Placozoa (e.g., Beagley et al. 1996; Dellaporta et al. 2006; Rot et al. 2006). Bilateria, however, (mostly) lack mitochondrial introns (see Vallès et al. 2008).

Mitochondrial introns in sponges are rare. Only three different types are known, all in the cytochrome oxidase subunit 1 (CO1) gene of tetillid Demospongiae (*Cinachyrella* and *Tetilla*), but also in plakinid Homoscleromorpha (*Plakina* and *Plakinastrella*) (Gazave et al. 2010; Rot et al. 2006; Szitenberg et al. 2010) (Fig. 1). All these introns harbor an ORF for a putative LAGLIDADG-type homing

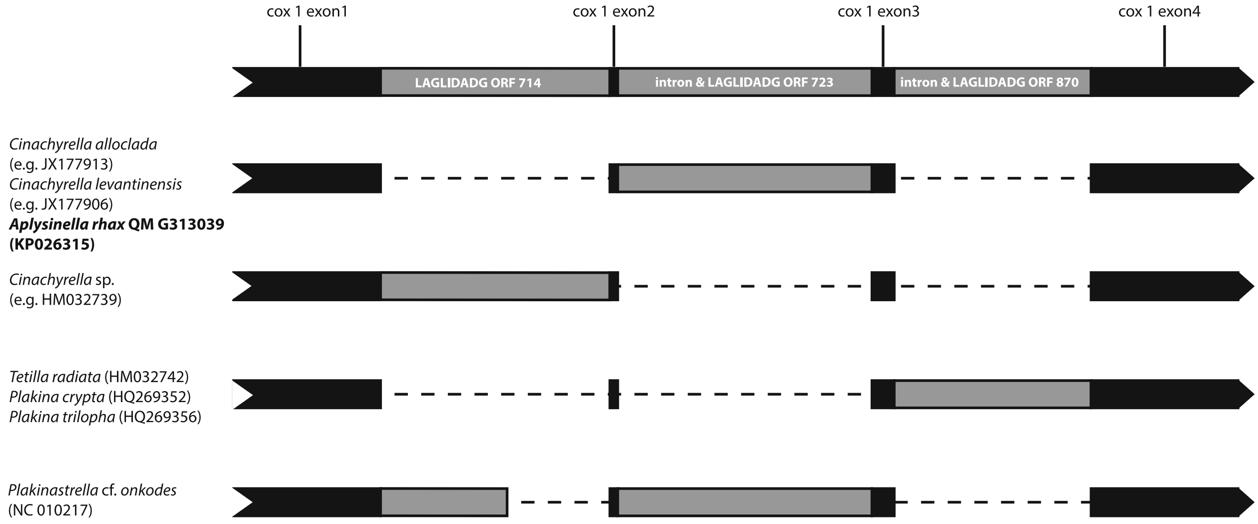


Fig. 1 Schematic view of the CO1 intron position in sponges. Three types of introns are known. They differ by their sequence and insertion points and are subsequently called "714", "723", and "870" following their distinct position relative to *Amphimedon queenslandica* COI (cf. Szitenberg et al. 2010). *P. onkodes* is the only sponge taxon with two introns, its "714" intron is short and lacks an ORF

Fig. 2 Maximum-likelihood tree of LAGLIDADG-type homing▶ endonuclease protein sequences from sponges, cnidarians, and non-metazoans from the published alignments (Rot et al. 2006; Szitenberg et al. 2010), and the partial sequences yielded from *Aplysinella rhax*. The data set is based on 80 protein characters from the 3′ region of *A. rhax* LAGLIDADG. The tree is reconstructed with RAxML with 100 rapid bootstraps and subsequent ML search under the PROTGAMMA model with MTREV. Numbers on the branches indicate bootstrap support (>65). Numbers preceding taxon names are Genbank accession numbers. Scale bar depicts substitutions per site

endonuclease. The high similarity of the introns (>85–99 %, Szitenberg et al. 2010) and their identical insertion site in CO1 are in strong contrast to the distant relationship of their hosts as Plakinidae and Tetillidae are members of two distant poriferan classes (see e.g., Wörheide et al. 2012). Insertions of LAGLIDADG-ORF introns into genes are sequence-tolerant, but site-specific (Edgell 2009), which complicate our understanding whether their patchy distribution derives from vertical transmission among ancestral sponge lineages with subsequent loss (Wang and Lavrov 2008), or from horizontal transmission (Haugen et al. 2005; Rot et al. 2006), or from a combination of both (see also Fukami et al. 2007; Szitenberg et al. 2010). We now detected a CO1 intron in a second demosponge lineage, Verongida, which corroborates the theory of combined vertical and horizontal transmission in sponges, but impede the use of popular CO1 primers.

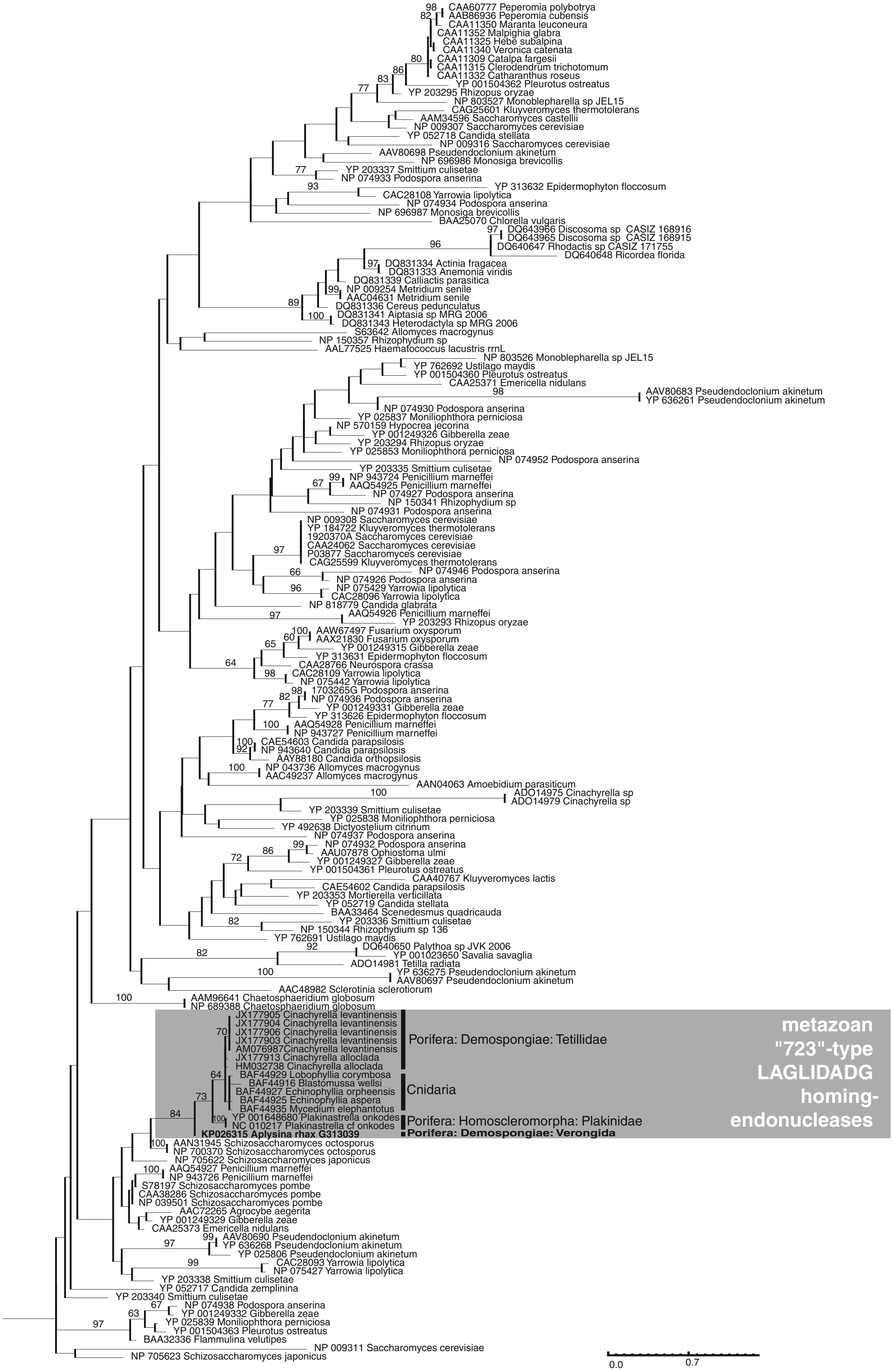
Materials and Methods

DNA of *Aplysinella rhax* (De Laubenfels 1954) (Queensland Museum voucher QMG313039) was extracted, and CO1 sequenced following previously published methods (Erpenbeck et al. 2012) using the reverse primer COX1-R1 (Rot et al. 2006) and 1:30 min PCR extension time (Genbank Acc. No: KP026315). Sequence annotation and alignments were performed with MacClade 4.08 (Maddison et al. 1999) and Geneious v6.1.6 (Biomatters). LAGLIDADG-type homing endonuclease protein sequences from published alignments (Rot et al. 2006; Szitenberg et al. 2010) and *Aplysinella rhax* were realigned with MAFFT v7.149b (G-INS-i Katoh and Standley 2013), and end-trimmed prior to phylogenetic reconstruction with RAxML v8.0.26 (Stamatakis 2006) (see Fig. 2 for details).

Results

CO1 of *Aplysinella rhax* possesses an intron at a similar position to *Cinachyrella alloclada* (Uliczka 1929), *C. levantinensis* Vacelet et al. 2007 and *Plakinastrella onkodes* (Uliczka 1929) (Fig. 1), and resembles their "723"-type LAGLIDADG homing endonuclease (cf. Szitenberg et al. 2010) with $\geq$80 % nucleotide similarity. Interestingly, p-distances of *Cinachyrella* sequences to the homoscleromorph *P. onkodes* are smaller (0.118) than to its fellow Demospongiae *A. rhax* (0.188). Similarly, the 3′intron region between the LAGLIDADG-ORF stop codon and the CO1 exon 3 (113 bp) of *Cinachyrella* spp. is 70 % identical to *P. onkodes*, but barely alignable to *A. rhax*.

The Maximum-likelihood analysis recovered all metazoan "723"-type CO1 introns as monophyletic (Fig. 2). The sponge sequences, however, are paraphyletic with


CAA60777 Peperomia polybotrya
AAB86936 Peperomia cubensis
CAA11350 Maranta leuconeura
CAA11352 Malpighia glabra
CAA11325 Hebe subalpina
CAA11340 Veronica catenata
CAA11309 Catalpa fargesii
CAA11315 Clerodendrum trichotomum
CAA11332 Catharanthus roseus
YP 001504362 Pleurotus ostreatus
YP 203295 Rhizopus oryzae
NP 803527 Monoblepharella sp JEL15
CAG25601 Kluyveromyces thermotolerans
AAM34596 Saccharomyces castellii
NP 009307 Saccharomyces cerevisiae
YP 052718 Candida stellata
NP 009316 Saccharomyces cerevisiae
AAV80698 Pseudendoclonium akinetum
NP 696986 Monosiga brevicollis
YP 203337 Smittium culisetae
NP 074933 Podospora anserina
YP 313632 Epidermophyton floccosum
CAC28108 Yarrowia lipolytica
NP 074934 Podospora anserina
NP 696987 Monosiga brevicollis
BAA25070 Chlorella vulgaris
DQ643966 Discosoma sp CASIZ 168916
DQ643965 Discosoma sp CASIZ 168915
DQ640647 Rhodactis sp CASIZ 171755
DQ640648 Ricordea florida
DQ831334 Actinia fragacea
DQ831333 Anemonia viridis
DQ831339 Calliactis parasitica
NP 009254 Metridium senile
AAC04631 Metridium senile
DQ831336 Cereus pedunculatus
DQ831341 Aiptasia sp MRG 2006
DQ831343 Heterodactyla sp MRG 2006
S63642 Allomyces macrogynus
NP 150357 Rhizophydium sp
AAL77525 Haematococcus lacustris rrnL
NP 803526 Monoblepharella sp JEL15
YP 762692 Ustilago maydis
YP 001504360 Pleurotus ostreatus
CAA25371 Emericella nidulans
AAV80683 Pseudendoclonium akinetum
YP 636261 Pseudendoclonium akinetum
NP 074930 Podospora anserina
YP 025837 Moniliophthora perniciosa
NP 570159 Hypocrea jecorina
YP 001249326 Gibberella zeae
YP 203294 Rhizopus oryzae
YP 025853 Moniliophthora perniciosa
NP 074952 Podospora anserina
YP 203335 Smittium culisetae
NP 943724 Penicillium marneffei
AAQ54925 Penicillium marneffei
NP 074927 Podospora anserina
NP 150341 Rhizophydium sp
NP 074931 Podospora anserina
NP 009308 Saccharomyces cerevisiae
YP 184722 Kluyveromyces thermotolerans
1920370A Saccharomyces cerevisiae
CAA24062 Saccharomyces cerevisiae
P03877 Saccharomyces cerevisiae
CAG25599 Kluyveromyces thermotolerans
NP 074946 Podospora anserina
NP 074926 Podospora anserina
NP 075429 Yarrowia lipolytica
CAC28096 Yarrowia lipolytica
NP 818779 Candida glabrata
AAQ54926 Penicillium marneffei
YP 203293 Rhizopus oryzae
AAW67497 Fusarium oxysporum
AAX21830 Fusarium oxysporum
YP 001249315 Gibberella zeae
YP 313631 Epidermophyton floccosum
CAA28766 Neurospora crassa
CAC28109 Yarrowia lipolytica
NP 075442 Yarrowia lipolytica
1703265G Podospora anserina
NP 074936 Podospora anserina
YP 001249331 Gibberella zeae
YP 313626 Epidermophyton floccosum
AAQ54928 Penicillium marneffei
NP 943727 Penicillium marneffei
CAE54603 Candida parapsilosis
NP 943640 Candida parapsilosis
AAY88180 Candida orthopsilosis
NP 043736 Allomyces macrogynus
AAC49237 Allomyces macrogynus
AAN04063 Amoebidium parasiticum
ADO14975 Cinachyrella sp
ADO14979 Cinachyrella sp
YP 203339 Smittium culisetae
YP 025838 Moniliophthora perniciosa
YP 492638 Dictyostelium citrinum
NP 074937 Podospora anserina
NP 074932 Podospora anserina
AAU07878 Ophiostoma ulmi
YP 001249327 Gibberella zeae
YP 001504361 Pleurotus ostreatus
CAA40767 Kluyveromyces lactis
CAE54602 Candida parapsilosis
YP 203353 Mortierella verticillata
YP 052719 Candida stellata
BAA33464 Scenedesmus quadricauda
YP 203336 Smittium culisetae
NP 150344 Rhizophydium sp 136
YP 762691 Ustilago maydis
DQ640650 Palythoa sp JVK 2006
YP 001023650 Savalia savaglia
ADO14981 Tetilla radiata
YP 636275 Pseudendoclonium akinetum
AAV80697 Pseudendoclonium akinetum
AAC48982 Sclerotinia sclerotiorum
AAM96641 Chaetosphaeridium globosum
NP 689388 Chaetosphaeridium globosum
JX177905 Cinachyrella levantinensis
JX177904 Cinachyrella levantinensis
JX177906 Cinachyrella levantinensis
JX177903 Cinachyrella levantinensis
AM076987Cinachyrella levantinensis
JX177913 Cinachyrella alloclada
HM032738 Cinachyrella alloclada
BAF44929 Lobophyllia corymbosa
BAF44916 Blastomussa wellsi
BAF44927 Echinophyllia orpheensis
BAF44925 Echinophyllia aspera
BAF44935 Mycedium elephantotus
YP 001648680 Plakinastrella onkodes
NC 010217 Plakinastrella cf onkodes
KP026315 Aplysina rhax G313039
Porifera: Demospongiae: Tetillidae
Cnidaria
Porifera: Homoscleromorpha: Plakinidae
Porifera: Demospongiae: Verongida
metazoan "723"-type LAGLIDADG homing-endonucleases
AAN31945 Schizosaccharomyces octosporus
NP 700370 Schizosaccharomyces octosporus
NP 705622 Schizosaccharomyces japonicus
AAQ54927 Penicillium marneffei
NP 943726 Penicillium marneffei
S78197 Schizosaccharomyces pombe
CAA38286 Schizosaccharomyces pombe
NP 039501 Schizosaccharomyces pombe
AAC72265 Agrocybe aegerita
YP 001249329 Gibberella zeae
CAA25373 Emericella nidulans
AAV80690 Pseudendoclonium akinetum
YP 636268 Pseudendoclonium akinetum
YP 025806 Pseudendoclonium akinetum
CAC28093 Yarrowia lipolytica
NP 075427 Yarrowia lipolytica
YP 203338 Smittium culisetae
YP 052717 Candida zemplinina
YP 203340 Smittium culisetae
NP 074938 Podospora anserina
YP 001249332 Gibberella zeae
YP 025839 Moniliophthora perniciosa
YP 001504363 Pleurotus ostreatus
BAA32336 Flammulina velutipes
NP 009311 Saccharomyces cerevisiae
NP 705623 Schizosaccharomyces japonicus
0.0
0.7

A. rhax as sister group to all other Metazoa. The cnidarian sequences are nested within the remaining sponges as sister group to the Tetillidae.

Discussion

The specificity of a mobile intron for insertion into homologous sites leads to rapid insertions at identical positions (see Haugen et al. 2005). Unlike e.g., SINE insertions, which may constitute potent phylogenetic markers (see also Miyamoto 1999), evolutionary tracking of group I introns remains more challenging. The initial interpretation of mt-introns in sponges as the result of a horizontal transfer from fungi (Rot et al. 2006) was subsequently doubted after homoscleromorph sponge (Wang and Lavrov 2008), and coral (Fukami et al. 2007) introns from the same sites were discovered. Instead, the patchy distribution of introns among poriferans in two families of different classes was interpreted with multiple losses of an ancestral intron (Wang and Lavrov 2008), as known among Bilateria (Cho et al. 2004). However, the *A. rhax* intron phenetic discrepancies in coding and noncoding regions and the resulting phylogenetic position of its protein are strongly incongruent with the currently accepted sponge systematics, and therefore oppose this scenario (Redmond et al. 2013; Wörheide et al. 2012). Instead, our discovery of a second intron-bearing lineage in demosponges supports a combination of vertical and horizontal transmissions (cf. Szitenberg et al. 2010). So far, complete co-speciation between the introns and their hosts, a prerequisite for the multiple-loss hypothesis could never be shown, therefore some lineages must have acquired introns independently (Fukami et al. 2007; Szitenberg et al. 2010). Group I introns with homing endonuclease likewise can invade each other or invade a “naked” intron without endonuclease ORF (Haugen et al. 2005). The horizontal transmission (see e.g., Vallès et al. 2008) with fixation will remain a rare event in metazoans in comparison to other opisthokonts as metazoan germ lines are relatively segregated, which inhibits the easy incorporation of DNA fragments (Burt and Koufopanou 2004).

The poriferan mitochondrial introns are inserted in the 5′region of CO1, a popular marker for phylogenetic analyses (e.g., Erpenbeck et al. 2007), and therefore hamper experiments by disrupting annealing sites of popular universal CO1 (barcoding) primers (e.g., Rot et al. 2006). Our findings that demosponge mt-introns are not restricted to Tetillidae (one of 97 demosponge families, Van Soest et al. 2012), but may be distributed among other demosponge lineages, must subsequently be considered for standardized mt-studies (e.g., DNA barcoding). The ~100 demosponge mt-genomes sequenced so far still fail to provide sufficient overview on intron distribution among the >8,500 sponge species for the identification of intron “hotspots” (Szitenberg et al. 2010) such as Tetillidae and Plakinidae.

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Conflict of interest The authors declare that they have no conflict of interest.

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