Using ITS1 and ITS2 to delineate Ctenophora as the sister to all Metazoans.

Monica E. Brown[1]
[1]Program in Microbiology, Iowa State University, Ames, IA, USA

ABSTRACT

Early animal evolution of four phyla: Choanoflagellates, Porifera, Ctenophora, and Cnidaria is commonly debated amongst evolutionary biologists. Using the ITS region sequences of 68 different species, the Porifera-sister and Ctenophora-sister hypotheses were tested. Data were collected from GenBank and then the programs MAFFT and RAxML were used to analyze data through bootstrap and branch length analysis. It was found that four genera need further research to possible reclassification as well as the finding in support of the Ctenophora-sister hypothesis. This indicates that the phylum Ctenophora is supported as the sister of all Metazoans, denoting further neural tissue development and body plan evolution hypotheses can be tested with this phylogeny in mind.

INTRODUCTION

Early animal evolution is a controversial subject amongst many evolutionary biologists. Four phyla are most frequently referred to: Choanoflagellates, Porifera, Ctenophora, and Cnidaria. Commonly, Porifera are known as sponges; Ctenophora are known as comb jellies; and Cnidaria are known as jellyfish. The latter three phyla are often debated regarding which is most closely related to the ancestor of all Metazoans, while Choanoflaellates are already commonly considered an outgroup. There are two main schools of thought, that of the Porifera-sister hypothesis (1) where the phylum Porifera is the sister group to all Metazoans and the second, more-controversial, hypothesis of Ctenophora-sister (2-5), where the phylum Ctenophora is the sister group to all Metazoans (Figure 1). This controversy stems partially from the naïve assumption that animals become more 'complicated' through evolution, whereas the simplest

phylum should be placed as the sister group to all Metazoans. Most recently, the concept of

Ctenophora being the sister group arose due to phylogenetic support (2-5). Likewise, debate over

the origin of neural tissues is common, with debate centered around whether (i) neural tissues or

cell types evolved once in the tree stem and then was subsequently lost in Porifera and the

phylum Placozoa, or (ii) neural tissues or cell types arose independently in two phyla, once in

Ctenophora and once in Cnidaria (6, 7). While establishment of which neural origin is not

considered in this paper, placement of either Porifera or Ctenophora will drive further research

towards the development of support for one tissue development hypotheses.
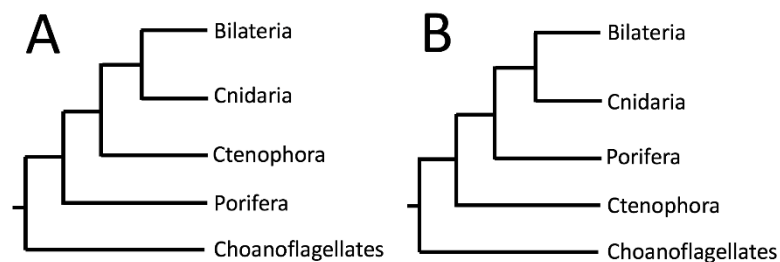
FIGURE 1 Alternative hypotheses from previous studies. (A) Porifera–sister hypothesis (B)

Ctenophora–sister hypothesis.

Research additionally has reported the successful use of the internal transcribed spacer

(ITS) region, which consists of ITS1, 5.8S ribosomal DNA (rDNA), and ITS2. As rDNA tends to

be very conservative, as noted with bacterial and archaeal lineages being elucidated through 16S

rDNA (8), the 5.8S rDNA sequence within the ITS region is useful for deep-rooted phylogenies

(9-11). One major problem of rDNA sequences involving phylogenies is the inability to clarify

differences at the species and sometimes the genus level due to low mutation/evolution rates (8).

One way this can be solved is the addition of the ITS1 and ITS2 sequences. Both of these regions

have extremely fast mutation/evolution rates, making them ideal for species illumination (12).

Moreover, these regions tend to be conservative in length but with the added bonus of tending to

organize in a tandemly repeated manner, meaning that their general size and organization remains the same, but there is high site mutation, making these sequences easily aligned across phyla (13). Also, it has been noted that the ITS region can be used to estimate phylogenetic relationships at the species level while maintaining the same branching order as other DNA regions determine, indicating that these regions do contain accuracy for phylogeny determination (13, 14).

Here, the Ctenophora-sister hypothesis will be tested to determine the sister group to all animals. In addition, this is the first time the Ctenophora phylum and Porifera phylum have been analyzed with this many species using the ITS region.

METHODS

Dataset Assembly. Complete DNA sequences of internal transcribed spacer 1 (ITS1), 5.8S, and internal transcribed spacer 2 (ITS2) rRNA, and partial DNA sequences of the small sub-unit (18S) and large sub-unit (28S) rRNA of 68 selected species were used to construct alignments and phylogenies (15). Due to limited resources, each sequence region was concatenated prior to data collection, leading to an inability to separately align ITS1, 5.8S, and ITS2. Selected sequences include 23 Ctenophora, 37 Porifera, 7 Cnidaria, and 1 Choanoflagellate. All data was downloaded from GenBank (15), the NIH genetic sequence database, and accession numbers are located in Table S1.

Alignment. Sequences were aligned using MAFFT (16) version 7.245 using localpair iterative refinement method (L-INS-i) with a maxiterate of 16 and input order-phylip output. Script available in Figure S1.

Maximum Likelihood. Alignments were analyzed using RAxML (17) version 8.2.9 using the GTR+Γ model with 30 stepwise additions and using *Salpingoeca kvevrii* as an outgroup to root

the tree. The GTR+Γ model is suited to DNA data. In addition, the alignments were analyzed

using the same software and model to generate 30 maximum likelihood trees on distinct starting

trees with the best likelihood saved with the same outgroup chosen to root the tree. Script

available in Figure S1.

Bootstrapping and Branch Length. Alignments were analyzed for bootstrap values using the

GTR+Γ and GTR+CAT models and a random seed number of 12345 and 100 replicates was

used. The GTR+CAT model is considered the most suitable model to use when looking at deep

phylogeny and ancestral groups as it accounts for site heterogeneity (5, 18, 19). Bipartitions and

branch lengths were then drawn on the best maximum likelihood tree and viewed using FigTree

version 1.4.3 (20). The tree is presented untransformed with branch lengths as well as

transformed using the function 'cladogram' and node labels of the bootstrap values due to

rooting of the tree with *Salpingoeca kvevrii.*

RESULTS

**Ctenophora is Sister to All Metazoans.**

Using complete ITS1, 5.8S and ITS2 sequences for 68 species, the tree topology for the phyla

Porifera, Ctenophora, and Cnidaria was determined and is presented in Figure 2. Figure 2

indicates that Ctenophora is the sister group to all other metazoans (red bar) as indicated by

being the first branch point as compared to the choanoflagellate outgroup (black arrow).
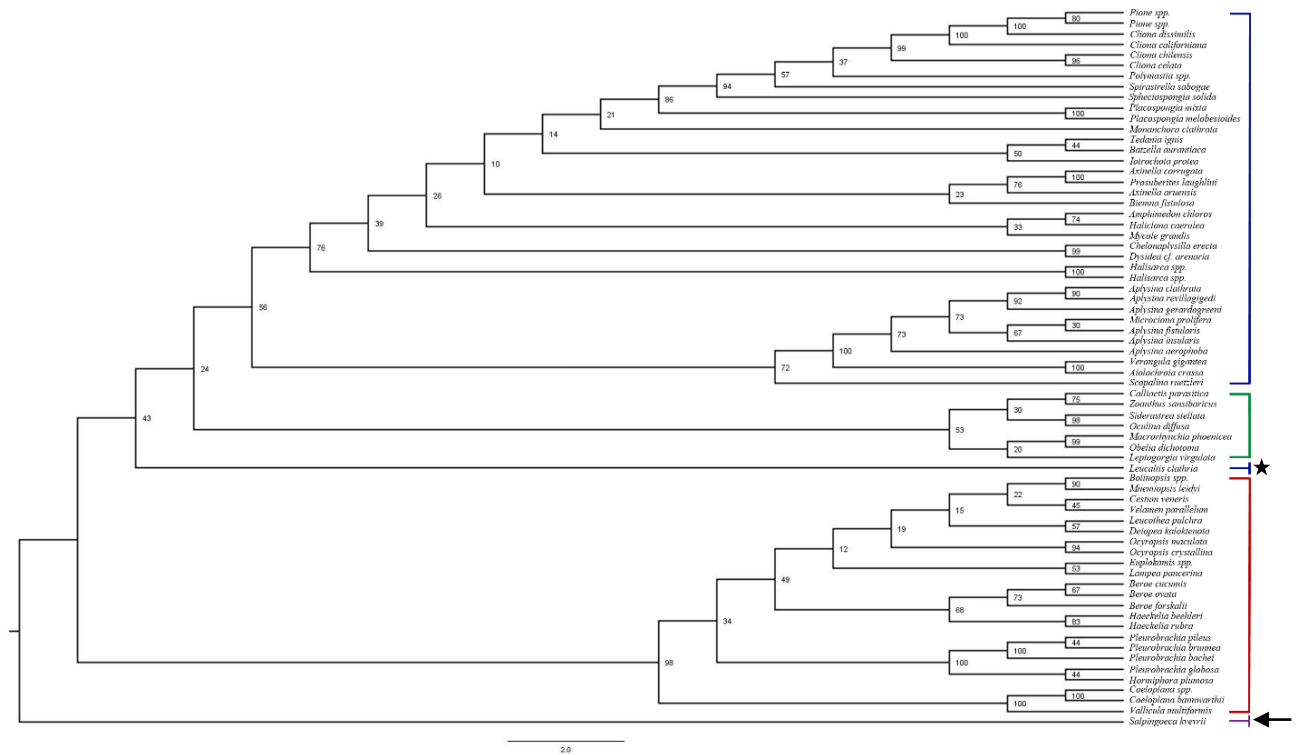
FIGURE 2 Bootstrap support value tree of all 68 species with the outgroup consisting of *Salpingoeca kvevrii,* a choanoflagellate. The black arrow (or purple bar) indicates the choanoflagellate outgroup, the red bar indicates the phylum Ctenophora, the green bar indicates the phylum Cnidaria, while the blue bars indicate the phylum Porifera. Larger image available in Figure S2.

Due to the use of ITS1 and ITS2, explanation of genera and species relationships

different to views currently held can be established. Three such discrepancies are clear in Figure

2, that of *Pione* and *Cliona*, *Axinella* and *Prosuberites*, as well as the species *Leucaltis clathria*.

The species *Leucaltis clathria* (black star in Figure 2), is found between the phyla Ctenophora

and Cnidaria. At first glance this seems like a misplacement, but in actuality this is due to being a

unique class as compared to all other sponges presented. To resolve this, additional species from

the same class should be added in future studies, but in the present study does not negate

findings. Looking at Figure 3, a closer view of the two remaining discrepancy locations with

bootstrap values from Figure 2 can be seen. In Figure 3A, there is an apparent identification that

Pione is a separate genus from *Cliona,* but complete bootstrap support indicate that these two genera actually may be the same genus, indicated by *Cliona dissimilis* and *Pione spp.*. Figure 3B also shows complete bootstrap support between *Axinella corrugata* and *Prosuberites laughlini,* indicating a possible need for the genus of *Prosuberites* to be combined with *Axinella*.
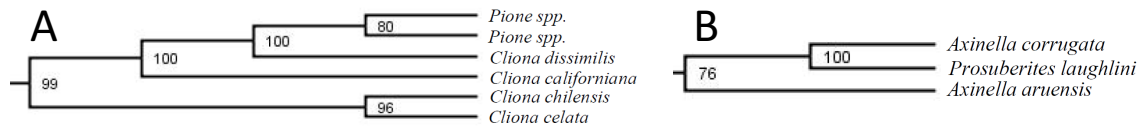
FIGURE 3 Close up of sponge species delineation by bootstrap from Figure 2. (A) The genera *Pione* and *Cliona*. (B) The genera *Axinella* and *Prosuberites*.

Though bootstrap support is one form of evidence, it alone is not enough to reconcile the need to combine two genera. An additional form of support can come in the form of branch lengths, which indicate the number of nucleotide substitutions per site. Figure 4 shows these branch lengths, where the smaller the branch length the less change/substitutions and the larger the branch length, the more change/substitutions.
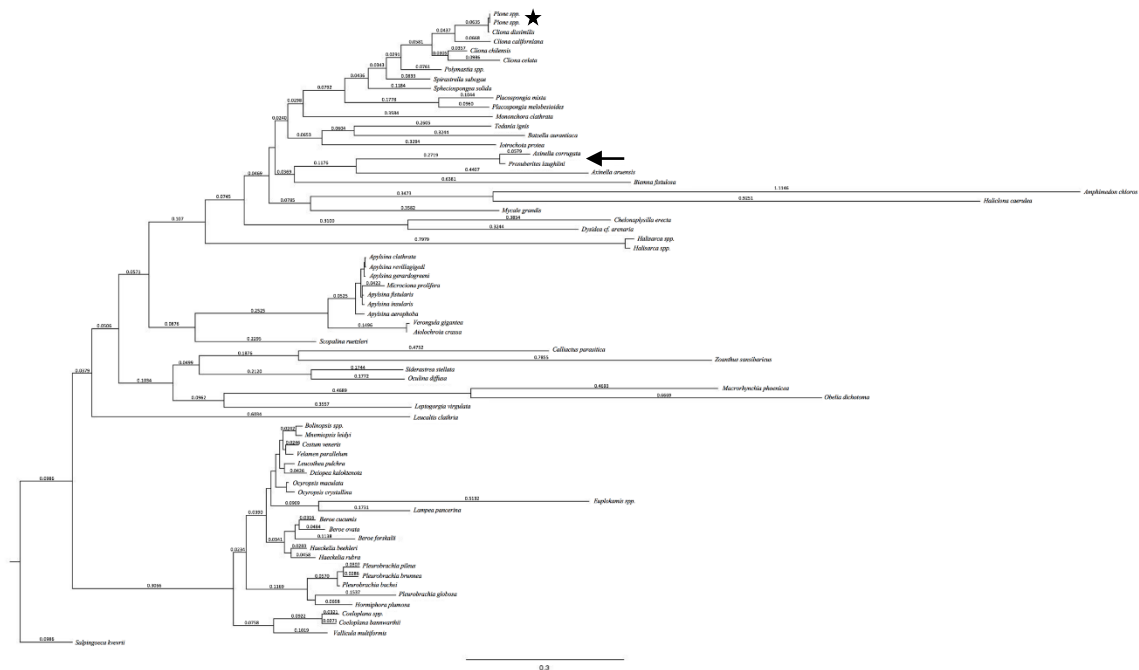
FIGURE 4 Phylogenetic tree with branch lengths indicating number of nucleotide substitutions per site. Branches without values denote lengths of 0.02 or less. Larger image available in Figure S3.

In regards to the reclassification of the genus *Pione* to *Cliona* and the genus *Prosuberites* to *Axinella*, the branch lengths give good support. For the genera *Pione* and *Cliona* (black star), the branch lengths are zero (not listed due to size constraints), signifying a very high chance that these two species are likely the same. In the case of *Prosuberites* and *Axinella* (black arrow), the branch lengths are larger than zero, but still small, which indicates that these two genera are highly related.

DISCUSSIONS AND CONCLUSIONS

Reclassification of Genera.

The phylogenetic analyses of the ITS1, 5.8S, and ITS2 region reveals a need for possible reclassification. The species *Prosuberites laughlini* and *Axinella corrugata* have complete bootstrap support values and relatively low branch lengths, indicating a high likelihood of a close relationship. Previous research also suggests that these sponges reside in similar regions, which could explain some of the misidentification (21). Further confirmation with more *Prosuberites spp.* and *Axinella spp.* with ITS region phylogenetic reconstruction is necessary before these can be placed in the same genus.

Additionally, the genera *Pione* and *Cliona* share numerous similarities. In common language, these genera are often confused with each other due to analogous characteristics. Furthermore, *Cliona dissimilis* and *Pione spp.* share complete bootstrap support as well as have branch lengths of zero. A branch length of zero indicates that there are no differences, genetically, between these species in the ITS region. Considering the variability of the ITS region, no differences is extremely uncommon which signifies that these species are likely the

same. The addition of complete bootstrap support further implicates that these genera, but especially species, could be the same. Additional phylogenetic support, such as Bayesian analysis, could further suggest the combination of these two genera into one, but should be carried out with other species to enumerate any differences between them to ensure this combination of genera is highly supported.

Through the use of the ITS region, differences between four genera in phylogenetic analyses indicate a need to further look into the classification of the genera *Cliona*, *Pione*, *Prosuberites*, and *Axinella*. While the analyses portrayed here are detailed, more support is necessary.

Ctenophora is the Sister to All Metazoans.

One major debate amongst evolutionary biologists is whether our most-recent common ancestor with Porifera is more- (Ctenophora-sister) or less- (Porifera-sister) recent than our most-recent common ancestor with Ctenophora. Based on the rDNA genes ITS1, 5.8S, and ITS2 and the analyses, the Ctenophora-sister hypothesis is most strongly supported. The use of the GTR+Γ and GTR+CAT models of maximum likelihood are previously strongly supported and resistant to systematic errors (22) and further supported with the addition of more taxa and different outgroups. The high bootstrap values and placement in relation to the outgroup Choanoflagellate, a known and commonly used outgroup for metazoan phylogenies, indicates support for the phylum Ctenophora as the sister to all metazoans. This Ctenophora-sister support makes major implications in early animal evolution, indicating neural tissues could arise in the tree stem in Ctenophora (23), and can further determine body plan evolution (1). While this may seem unlikely at first, in reality using complexity as a measure of character in evolution is flawed due to an inability to define what complexity is. Moreover, most models of the Porifera-sister

hypothesis use parsimonious methods, which, while useful in some scenarios, again defines that

complexity is a character and therefore the least complex should be at the base of early animal

evolution (24). It is known that convergent evolution is a relatively common phenomenon in

evolution, as this has been described for over a decade on eye evolution alone (25), so the

concept that neural and muscular systems also evolved through convergent evolution is not a

reach, implicating that neural tissues could arise in two separate lineages. Additionally, other

papers support that ctenophores are the sister to all Metazoans (26, 27). Through the use of

proper model selection and high bootstrap support, the overall findings presented here also

support that ctenophores are the sister to all Metazoans.

REFERENCES

1   **Dohrmann M, Wörheide G.** 2013. Novel scenarios of early animal evolution—Is it time to rewrite textbooks? *Integr Comp Biol* **53(3)**:503–511.

2   **Dunn CW, Hejnol A, Matus DQ, Pang K, Browne WE, Smith SA, Seaver E, Rouse GW, Obst M, Edgecombe GD, Sorensen MV, Haddock SHD, Schmidt-Rhaesa A, Okusu A, Kristensen RM, Wheeler WC, Martindale MQ, Giribet G.** 2008. Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* **452(7188)**:745–749.

3   **Hejnol A, Obst M, Stamatakis A, Ott M, Rouse GW, Edgecombe GD, Martinez P, Baguñà, Bailly X, Jondelius U, Wiens M, Müller WEG, Seaver E, Wheeler WC, Martindatle MQ, Giribet G, Dunn CW.** 2009. Assessing the root of bilaterian animals with scalable phylogenomic models. *Proc Biol Sci* **276(1802)**:4261–4270.

4   **Moroz LL, Kocot KM, Citarella MR, Dosung S, Norekian TP, Povolotskaya IS, Grigorenko AP, Dailey C, Berezikov E, Buckley KM, Ptitsyn A, Reshetov D, Mukherjee K, Moroz TP, Bobkov YV, Swore JJ, Girardo DO, Fodor A, Gusev F, Sanford R, Bruders R, Kittler E, Mills CE, Rast JP, Derelle R, Solovyev VV, Kondrashov FA, Swalla BJ, Sweedler JV, Rogaev EI.** 2014. The ctenophore genome and the evolutionary origins of neural systems. *Nature* **510(7503)**:109–114.

5   **Nosenko T, Schreiber F, Adamska M, Adamski M, Eitel M, Hammel J, Maldonado M, Müller WEG, Nickel M, Schierwater B, Vacelet J, Weins M, Wörheide G.** 2013. Deep metazoan phylogeny: When different genes tell different stories. *Mol Phylogenet Evol* **67(1)**:223–233.

6   **Ryan JF, Chiodin M.** 2015. Where is my mind? How sponges and placozoans may have lost neural cell types. *Phil Trans R Soc B* **370**: 1-6.

7   **Ryan JF.** 2014. Did the ctenophore nervous system evolve independently? *Zoology* **117**: 225-226.

8   **Janda JM, Abbott SL.** 2007. 16S rRNA Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *J Clin Microbiol* **45(9)**: 2761-2764.

9   **Selker E, Yanofsky C.** 1979. Nucleotide sequence and conserved features of the 5.8S rRNA coding region of *Neurospora crassa*. *Nucleic Acids Res* **6(7)**: 2561-2567.

10   **Jobes DV, Thien LB.** 1997. A Conserved Motif in the 5.8S Ribosomal RNA (rRNA) Gene is a Useful Diagnostic Marker for Plant Internal Transcribed Spacer (ITS) Sequences. *Plant Mol Biol Reporter* **15(4)**: 326-334.

11   **Gillespie JJ, Johnston JS, Cannone JJ, Gutell RR.** 2006. Characteristics of the nuclear (18S, 5.8S, 28S, and 5S) and mitochondrial (12S and 16S) rRNA genes of *Apis mellifera* (Insecta: Hymenoptera): structure, organization, and retrotransposable elements. *Insect Mol Biol* **15(5)**: 657-686.

12   **Yao H, Song J, Liu C, Luo K, Han J, Li Y, Pang X, Xu H, Zhu Y, Xiao P, Chen S.** 2010. Use of ITS2 region as the universal DNA barcode for plants and animals. *PLoS ONE* **5(10)**: e13102.

13   **Coleman AW, Vacquier VD.** 2002. Exploring the Phylogenetic Utility of ITS Sequences for Animals: A Test Case for Abalone (***Haliotis***). *J of Mol Evol* **54(2)**: 246-257.

14   **Itskovich V, Glyzina O, Kaluzhnaya O.** 2017. Intraspecific and interspecific sequence variablitity in the ITS region of the rDNA of freshwater sponges of Lake Baikal and East Siberia. *Inland Waters* **7(3)**: 259-266.

15   **Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW.** 2016. GenBank. *Nucleic Acids Res* **44**: D67-D72.

16   **Katoh K, and Toh H.** 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* **9**: 286-298.

17   **Stamatakis A.** 2014. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* **30(9)**: 1312-1313.

18   **Halanych KM, ed Roubos EW, ed Wendelaar-Bonga SE, ed Vaudry H, ed De Loof A** 1999. Metazoan phylogeny and the shifting comparative framework. Recent Developments in Comparative Endocrinology and Neurobiology. *Shaker, Maastrict, The*

Netherlands: 3–7.

19 **Philippe H, Brinkmann H, Lavrov DV, Littlewood TJ, Manuel M, Wörheide G.** 2011. Resolving difficult phylogenetic questions: Why more sequences are not enough. *PLoS Biol* **9(3)**: e1000602.

20 **Rambaut A.** 2016. FigTree version 1.4.3. http://tree.bio.ed.ac.uk/software/figtree/

21 **Horton T, Kroh A, Ahyong S, Bailly N, Boury-Esnault N, et al.** (2018) World Register of Marine Species. http://www.marinespecies.org/

22 **Whelan N, Kocot KM, Moroz LL, Halanych KM.** 2015. Error, signal, and the placement of Ctenophora sister to all other animals. *PNAS* **112(18)**: 5773-5778.

23 **Jékely G, Paps J, and Nielsen C.** 2015. The phylogenetic position of ctenophores and the origin(s) of nervous systems. *EvoDevo* **6**:1.

24 **Simion P, Phillippe H, Baurain D, Jager M, Richter DJ, Di Franco A, Roure B, Satoh N, Quéinnic É, Ereskovsky A, Lapébie P, Corre E, Delsuc F, King N, Wörheide G, Manuel M.** 2017. A Large and Consistent Phylogenomic Dataset Supports Sponges as the Sister Group to All Other Animals. *Cell* **27(7)**: 958-957.

25 **Ogura A, Ikeo K, Gojobori T.** 2004. Comparative Analysis of Gene Expression for Convergent Evolution of Camera Eye Between Octopus and Human. *Genome Res* **14(8)**: 1555-1561.

26 **Halanych K.** 2015. The ctenophore lineage is older than sponges? That cannot be right! Or can it? *J of Exp Bio* **218**: 592-597.

27 **Whelan N, Kocot KM, Moroz TP, Mukherjee K, Williams P, Paulay G, Motoz LL, Halanych KM.** 2017. Ctenophore relationships and their placement as the sister group to all other animals. *Nature Ecology & Evolution* **1**: 1737-1746.