

Improved phylogenomic taxon sampling noticeably affects non-bilaterian relationships

Letter

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Abstract

Despite expanding data sets and advances in phylogenomic methods, deep level metazoan relationships remain highly controversial. Recent phylogenomic analyses depart from classical concepts in recovering ctenophores as the earliest-branching metazoan taxon, and propose a sister-group relationship between sponges and cnidarians (e.g. Dunn et al., 2008, *Nature* 452: 745). Here, we argue that these results are artefacts stemming from insufficient taxon sampling and long-branch attraction (LBA). By increasing taxon sampling from previously unsampled non-bilaterians and using an identical gene set to that reported by Dunn et al. (2008) we recover monophyletic Porifera as the sister group to all other Metazoa. This suggests that the basal position of the fast-evolving Ctenophora proposed by Dunn et al. was due to LBA and that broad taxon sampling is of fundamental importance to metazoan phylogenomic analyses. Additionally, saturation in the Dunn et al. character set is comparatively high, possibly contributing to the poor support for some non-bilaterian nodes.

Resolving the relationships of deep branching metazoan lineages is critical if we are to understand early animal evolution. Unravelling these relationships through the analysis of large scale molecular data sets has recently given birth to the field of phylogenomics (e.g. Philippe et al. 2005). Despite significant advances in this field, recent studies have generated contradictory results regarding relationships within and between early-diverging metazoan lineages: cnidarians, ctenophores (comb-jellies), sponges, placozoans (anatomically the simplest extant metazoans), and bilaterians. Placozoans have historically been regarded by some as relicts of the metazoan ancestor (see summary by Schierwater 2005), and some recent analyses place Placozoa at the base of a group of non-bilaterian animals (Dellaporta et al. 2006; Schierwater et al. 2009). However, recent whole genome (Srivastava et al. 2008) and phylogenomic (Philippe et al. 2009) analyses including *Trichoplax* recovered sponges as the sister-group to all other metazoans in accordance with morphological analyses (Ax 1996). Such contradictory hypotheses regarding non-bilaterian metazoan relationships prevent a consensus view of metazoan evolution, a goal that is of fundamental importance if we hope to fully understand the early evolution of animals (for an overview see Erpenbeck and Wörheide 2007).

A recent phylogenomic analysis adds further controversy to this debate (Dunn et al. 2008) (compare also Hejnol et al. 2009). Their outcome is highly unusual as sponges form a clade with the Cnidaria, while the ctenophores (despite being morphologically derived), are proposed to be the earliest branching metazoan taxon. As suggested by Philippe et al. (2009), we hypothesized that a long-branch attraction (LBA) artefact was responsible for

these controversial findings due to insufficient ingroup sampling and an inappropriate choice of outgroup taxa. Furthermore, the Placozoa are conspicuously absent from the Dunn et al. (2008) data set, and sponges are represented by only one Demospongiae and one Homoscleromorpha with no representatives of the remaining two extant sponge classes: Calcarea (Calcispongiae or calcareous sponges) and Hexactinellida (glass sponges). Sparse taxon sampling is a common pitfall of phylogenetic analyses (Lecointre et al. 1993) and is largely responsible for the lack of a robustly supported non-bilaterian metazoan phylogeny (Erpenbeck and Wörheide 2007). With a largely different gene set (only 45 genes in common with the 150 gene set of Dunn et al. 2008) and an increased sampling of non-bilaterian species, Philippe et al. (2009) obtained monophyletic sponges as the first-diverging metazoan lineage, and a sister-group relationship between the Cnidaria and the Ctenophora.

To test whether insufficient sampling of non-bilaterian taxa and inappropriate outgroup choice adversely influenced the analyses performed by Dunn et al. (2008), we re-analysed their 64-taxon matrix cleared of instable taxa (leaf stability < 90%) and with the following major modifications (cf. Baurain, Brinkmann, and Philippe 2007):

- 1) Ingroup taxon sampling was increased by the addition of non-bilaterian EST and genomic sequences. These included: 12 additional sponge taxa representing all four major sponge lineages; one additional ctenophore; five additional cnidarians (see Supplementary Table 1) and *Trichoplax adhaerens* (Placozoa).

2) We removed outgroup taxa with long branches. Long branches in the outgroup can strongly influence the topology of early branching ingroups (Philippe and Laurent 1998; Rota-Stabelli and Telford 2008). The long branches of the fungal outgroup are not visible in the cladogram of the PhyloBayes analysis (CAT+ Γ 4) of Dunn et al. (see their Fig. 2), but are evident in their Supplementary Figure 1. Consequently, we analysed our dataset with two sets of outgroups. First using only choanoflagellates, the most likely sister-group to all Metazoa (Carr et al. 2008), consisting of *Monosiga ovata* (shortest branch of Dunn et al.'s outgroup taxa), *Monosiga brevicollis* (complete genome data) and *Proterospongia* sp.. Second, with more distant outgroups, such as those used by Dunn et al. (2008) (see Supplementary Figure 1, and Supplementary Data for a detailed taxon list and methods used).

Furthermore we eliminated errors (e.g., frameshifts) and refined the Dunn et al. (2008) alignment, e.g., by reducing missing data and removing 2,150 ambiguously aligned positions (see Supplementary Data for detailed procedures). Our extended data set with the choanoflagellate-only outgroup consists of 80 taxa and 19,002 characters. Using this dataset we performed Bayesian phylogenetic analyses under the CAT+ Γ 4 model (Lartillot and Philippe 2004) and subsequent non-parametric bootstrapping (cf. Philippe et al. 2009).

Contrary to Dunn et al. (2008), and also Hejnol et al. (2009), we recover sponges as the sister-group to all other metazoan taxa (Figure 1). This is in congruence with earlier morphological (Ax 1996), and phylogenomic analyses

(Philippe et al. 2009). In accordance with the latter study, we also recover sponges as a monophyletic group. The Homoscleromorpha, a taxon previously assigned to the Demospongiae (see Hooper and Van Soest 2002), are found to be the sister-group to Calcarea as suggested by van Soest (1984) and Grothe (1989) based on morphology, and subsequently by Dohrmann et al. (2008) based on rRNA data. Similarly, Hexactinellida and the remaining Demospongiae *sensu stricto* form a monophyletic group (Silicea *sensu stricto*).

The basal position of ctenophores proposed by Dunn et al. (2008) was probably caused by the attraction of ctenophores to distant outgroup species, particularly fungi. In comparison, our re-analysis of the updated Dunn et al. (2008) dataset with increased ingroup taxon-sampling and a refined alignment indicates that LBA is reduced, independent of whether we use the choanoflagellate-only outgroup or more distant outgroups (see Fig. 1 and Supplementary Figure 1). This indicates that ingroup-taxon sampling, and probably to a lesser extent data refinement, are the most important parameters affecting non-bilaterian relationships.

Results of our analyses indicate that sponges are the sister group to the remaining Metazoa, and Placozoa are sister to the Bilateria. We also recover both monophyletic Ctenophores and Cnidaria, but they are paraphyletic with respect to Placozoa+Bilateria (Fig. 1). This is in contrast to the findings of Philippe et al. (2009) that supported the "Coelenterata hypothesis" (c. f. Haeckel 1866), i.e., a monophyletic Cnidaria+Ctenophora clade and a sister-group relationship between Coelenterata and Bilateria. However, support

values for the position of Ctenophora, Cnidaria and Placozoa in our analysis are either not significant (posterior probabilities < 0.9) or low (bootstrap support < 70%). We suspected that Dunn et al.'s character set contains a substantial amount of non-phylogenetic signal due to multiple substitutions. To test this, we conducted a saturation analysis of inferred substitutions against observed amino acid differences (Figure 2). This revealed a higher saturation in the original Dunn et al. (2008) character set (slope = 0.38x) compared to the Philippe et al. (2009) character set (slope = 0.46x) (Figure 2). From this we conclude that despite increasing the number of non-bilaterian taxa by a factor of 3 (from 9 to 27), multiple substitutions have partly masked phylogenetic signal contributing to the incongruent results reported here with those of Philippe et al. (2009). However, with the expanded and refined dataset reported here none of these incongruencies are statistically significant, indicating that non-phylogenetic signal has been reduced with respect to the original character set of Dunn et al. (2008). Furthermore, Dunn et al. (2008) recovered high support for the sister-group relationship of ctenophores to the remaining Metazoa – based on our analyses here this hypothesis should be rejected (with a bootstrap value of 91%).

The inclusion of additional taxa has little influence on the relationships within and between bilaterian crown groups. Three of the four differences between the findings of Dunn et al. (2008) and our results affect the relationships of a single sequence within their well-defined clades (*Euprymna* within Mollusca, *Paraplanocera* within Platyhelminthes and *Anoplodactylus* among the chelicerate arthropods). None of these splits were strongly supported in the original Dunn et al. (2008) analysis. Additionally, we do not recover

Panarthropoda due to a difference in the position of Tardigrada.

Panarthropoda was also weakly supported in the Dunn et al. (2008) analysis (PP values under WAG and CAT models were 0 and 0.86 respectively, and RAxML bootstrap support under the WAG model with 64 and 77 taxa was 4% and 2% respectively).

Our results highlight the sensitivity of phylogenomic studies to ingroup taxon sampling, and demonstrate the need for great care in the analysis and interpretation of large data sets. Character-rich analyses are thought to outperform character-poor analyses, and have been suggested to be of greater importance than increased taxon sampling with regard to recovering robust metazoan phylogenies (Rokas and Carroll 2005). However, our analyses demonstrate the strong influence of taxon sampling, even though non-bilaterian taxa still remain under-represented (Cnidaria: no Octocorallia, Ceriantharia, Cubozoa or Staurozoa; Ctenophora: no Platyctenida, Beroida, Cestida; just one placozoan strain etc.). The phylogenomic approach promises to reveal a well resolved consensus metazoan tree, but it should not be assumed that a large dataset will automatically produce a strong or correct phylogenetic signal (Jeffroy et al. 2006). A wide range of factors, such as saturation, LBA, the best fitting evolutionary model and appropriate outgroup choice (Philippe et al. 2005) need to be carefully addressed before a fully resolved and robust animal tree of life will be realized.

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Figures:

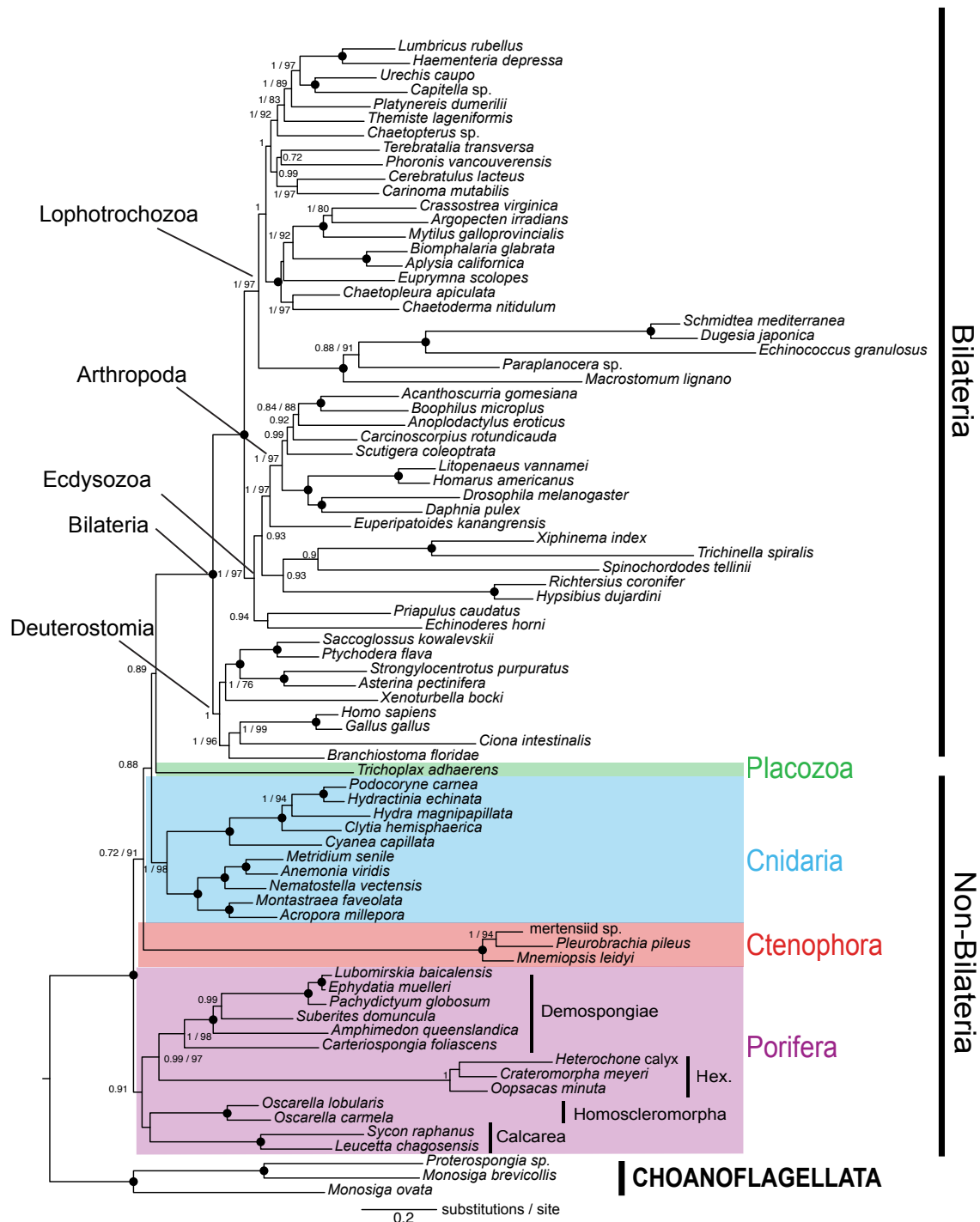


Figure 1: Phylogenetic tree based on refinements to the Dunn et al. (2008) 64-taxon set reconstructed with PhyloBayes (Lartillot, Lepage, and Blanquart 2009) under the CAT+ Γ 4 model. Choanoflagellates were set as outgroup and an additional 18 non-bilaterian taxa included. Posterior probabilities > 0.7 are indicated followed by bootstrap support values > 70. A large black dot indicates maximum support in posterior probabilities and bayesian bootstraps (= 1 / 100).

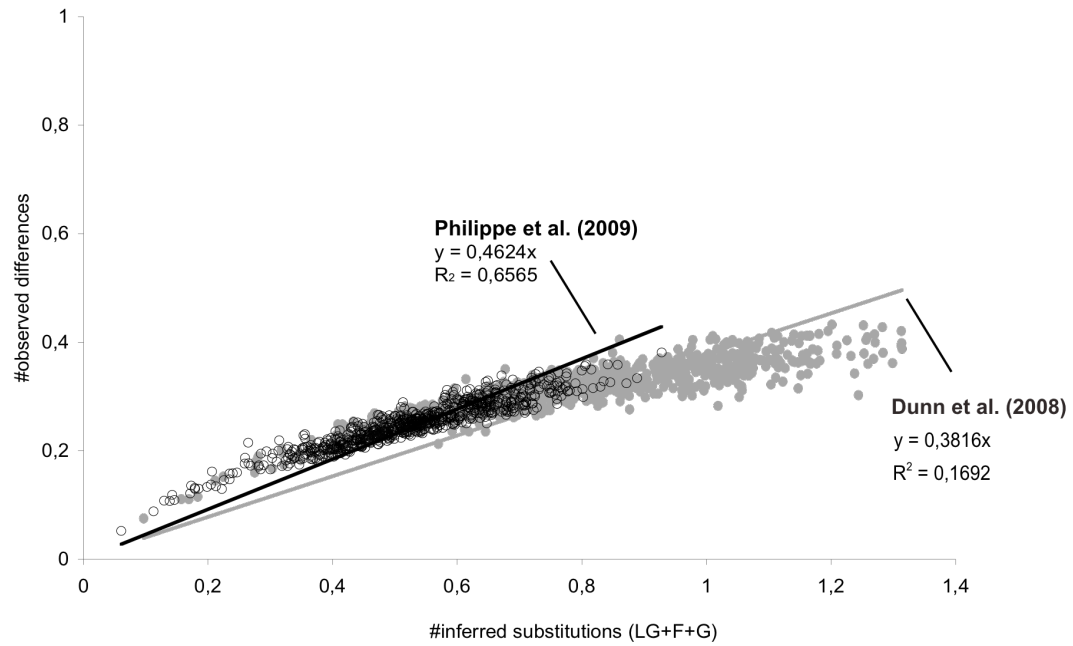


Figure 2: Saturation plot of character sets. See supplementary materials for method details. Grey line and filled dots: Dunn et al. (2008). Black line and open dots: Philippe et al. (2009).