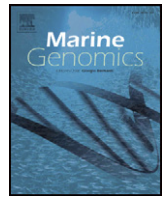




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Letter to the Editor

A reply to Medina et al. (2011): Crawling through time: Transition of snails to slugs dating back to the Paleozoic based on mitochondrial phylogenomics

The most recent approach on opisthobranch phylogeny by Medina et al. (2011) merits both credit and criticism. On one hand, it contributes new and valuable data; no less than 13 complete euthyneuran gastropod mitochondrial genomes were sequenced, and concatenated amino acids were analyzed in a total set of 25 gastropods. The phylogenetic tree resulting from maximum likelihood analyses is fully resolved, and the support for non-critical nodes was very high. However, there are a number of significant problems that arise in this paper, that need to be discussed.

Medina et al. (2011) assert that their paper substantiates mitogenomic approaches as a reliable approach to solving difficult phylogenetic problems or nodes, and positively reviews similar earlier research (Grande et al., 2002, 2004a,b, 2008). Yet paradoxically, the cited studies showed high node support for alternative contradictory topologies. These unconventional relationships, e.g. Patellogastropoda as a maximally supported sister of Euthyneura (Grande et al., 2008), could easily be explained by limited taxon sampling. Earlier studies emphasized the potential power of apomorphies derived from mitochondrial gene rearrangements for euthyneuran systematics (e.g. Grande et al., 2002). Yet Medina et al. (2011) apparently found no major apomorphic gene rearrangements to support their topology; instead, they refer to an 'ancestral opisthobranch arrangement' without any explicit reconstruction given.

Fundamentally, the taxon sampling used by Medina et al. (2011) is still limited, highly selective, and based on the predefined concept of reciprocal monophyly of the traditional euthyneuran groups 'Opisthobranchia' and 'Pulmonata'. To represent the ingroup, the authors include six traditional opisthobranch orders, however, problematic major groups such as Tylodinoidea, Runcinoidea, Thecosomata, Gymnosomata, Acochloridia and Rhodopemorpha are lacking, and are crucial to testing monophyly of 'Opisthobranchia'. *A priori* definitions of 'Opisthobranchia' and 'Pulmonata' as monophyletic sister taxa contradict most, if not all, recent papers addressing heterobranch relationships. This includes those based on morphology (Wägele and Klussmann-Kolb, 2005) or molecular markers (Grande et al., 2008; Dinapoli and Klussmann-Kolb, 2010; Göbbeler and Klussmann-Kolb, 2010; Jörger et al., 2010; Dinapoli et al., 2011; all papers available prior to final submission of Medina et al., 2011), and also a recent molecular phylogeny by one of the authors (Dayrat et al., 2011). Apart from acteonoids resulting as opisthobranchs, and pyramidellids resulting as pulmonates, no lower heterobranchs (sensu Allogastropoda) were included, and further outgroups chosen are distant and unjustified. Arguably construed as a *posteriori* sampling selection, Medina et al. (2011) also discard several available pulmonate mitogenomes with no justification other

than claiming they show 'particularly long branches'. Our own preliminary amino acid alignments on all available gastropod genomes, however, do not show any specific irregularities within these taxa (own observations). It is a pity that Medina et al. (2011) do not discuss the changes in tree topology that these 'long branch taxa' might have caused, especially since monophyly of 'Pulmonata' and its potential sister group relationship to 'Opisthobranchia' is affected when these taxa are added (see Grande et al., 2008).

The outcome of this taxon selection regime is a euthyneuran tree topology that, taken alone and unrooted, shows some similarity to results from comprehensive multi-locus studies (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Dayrat et al., 2011). It still differs regarding the relative positions of e.g. Acteonoidea and Sacoglossa, and, in particular, the position of the euthyneuran root. While the sampling of 'lower heterobranch' outgroups has been dramatically improved in recent multi-locus studies, the analysis by Medina et al. (2011) still uses very distant caenogastropod outgroups. Unsurprisingly, and undiscussed by Medina et al. (2011), phylogenetic distance is reflected by the long caenogastropod and euthyneuran stem lines, and long pulmonate branches. Compared with well-rooted topologies from recent multi-locus studies (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010), long branches and misrooting are likely responsible for Medina et al.'s (2011) euthyneuran bifurcation into 'monophyletic' Opisthobranchia and Pulmonata. These taxa were, however, made monophyletic only by redefinition.

A key problem to the interpretation of the topology presented by Medina et al. (2011) remains with the appearance of the pulmonate *Siphonaria* among opisthobranch clades. Although re-defining morphological features of *Siphonaria* to a supposedly opisthobranch relationship, the authors did not discuss any contradictory evidences, e.g. topologies of recent multi-locus studies (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Dayrat et al., 2011), records of closable pneumostomes from some siphonariids (e.g. Marshall and McQuaid, 1992), or several other traditional putative synapomorphies for pulmonates that are also present in *Siphonaria*, such as cerebral glands, mediodorsal bodies, and a procerebrum with double connectives (e.g. Van Mol, 1967; Saleuddin et al., 1997). Ignoring older ideas that pulmonary and mantle cavities of pulmonates and opisthobranchs are homologous (Ruthensteiner, 1997) and a recent reclassification of Euthyneura with *Siphonaria* as a basal (pan)pulmonate (Jörger et al., 2010), the authors missed hypotheses that explain both the (plesiomorphic) similarity of *Siphonaria* with (eu)opisthobranchs and the presence of (possibly also plesiomorphic) 'pulmonate features'. Instead, 'Opisthobranchia' sensu Medina et al. (2011) is maintained as monophyletic only by briefly reinterpreting the morphology of *Siphonaria* to fit into their concept of 'Opisthobranchia'. Furthermore, in conflict with most modern literature (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Valdés et al., 2010), Acteonoidea are treated as opisthobranchs by Medina et al. (2011) without any discussion. The same applies for the formerly 'lower heterobranch' pyramidellid *Pyramidella* nesting within 'Pulmonata'; a typical result in recent molecular studies (Klussmann-Kolb et al., 2008; Dinapoli and

Klussmann-Kolb, 2010; Jörger et al., 2010; Dayrat et al., 2011; Dinapoli et al., 2011), but this fact remains unaddressed by the authors. Most tellingly, 'Opisthobranchia' and 'Pulmonata' sensu Medina et al. (2011) are the only clades that do not garner any bootstrap support. We suggest that they may instead represent artificial assemblages of heterobranch taxa, as indicated by recent direct sequencing approaches on much more representative taxon sets (Dinapoli and Klussmann-Kolb, 2010; Göbbeler and Klussmann-Kolb, 2010; Jörger et al., 2010; Dinapoli et al., 2011).

Instead of acknowledging limitations regarding study design and outcomes, Medina et al. (2011) choose to name major clades, which is usually seen as a proxy for confidence in such hypotheses. The clade Acteopleura (Acteonoidea plus Nudipleura) had resulted from earlier direct sequencing analyses (Vonnemann et al., 2005), but was shown to be an artifact of limited taxon sampling in subsequent studies using a broader set of lower heterobranchs (*Rissoella*, not Nudipleura, is sister to Acteonoidea; Göbbeler and Klussmann-Kolb, 2010; Jörger et al., 2010). Their clade Siphoglossa (*Sacoglossa* plus *Siphonaria*) also resulted previously from analyses with more representative taxon sets (Jörger et al., 2010), but has never achieved any bootstrap support in past or current maximum likelihood analyses, and must be considered dubious. Finally, the clade Placoesophaga (Cephalaspidea plus Anaspidea), as defined by the esophageal cuticle, is synonymous to Euopisthobranchia sensu Jörger et al. (2010).

Medina et al. (2011) were the first to run molecular clock analyses for gastropods using whole mitochondrial genomes and recover a much earlier origin of euthyneuran, pulmonate and opisthobranch gastropods than suspected from fossils and earlier molecular clock approaches on multi-locus datasets (Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010). Rather than suggesting their limited data set and resulting topology is responsible for unrealistic (as compared to current knowledge on fossil record) time estimates, this discrepancy was explained by the generally poor heterobranch fossil record and earlier miscalibrations. However, Medina et al. (2011) apparently miscalibrated their own analysis using first appearances of potential stemline fossils as minimal node ages, i.e. divergence of extant members of these clades (see Jörger et al., 2010 for details). The same misunderstanding occurred when criticizing Dinapoli and Klussmann-Kolb (2010) for apparently much too recent ages of 'Siphonariidae' (in fact two *Siphonaria* species, 22 Mya) and 'Cephalaspidea' (just two taxa, 49 Mya), while the oldest fossils date back to 161 and 208 Mya, respectively; the dated stemlines, however, fit quite well with such fossil ages.

Although the timing of divergence is a major point of Medina et al. (2011), their BEAST analysis is not adequately described, calibration priors were counter intuitively set as normal distributions (minimum clade ages are unlikely to be represented by a normal distribution), and apparently only a single run was attempted before being abandoned. For example, on page 53 the authors claim 'In the Bayesian analysis all nodes are supported by 100% posterior probability values', which is simply not true according to their Fig. 1. Also, since no Bayesian analysis apart from a BEAST run is mentioned in the Material and methods section, where do the Bayesian analysis and posterior probabilities mentioned in Fig. 1 come from? If they derive from an interrupted BEAST run not reaching convergence, these support values must be disregarded. Using R8s instead, Medina et al. (2011) dated the Euthyneura node, i.e. the divergence of extant euthyneuran taxa, at 523 Mya, in the early Cambrian period. Stemline euthyneurans (or stem heterobranchs, since Acteonoidea are included) then must be much older – perhaps more than a billion years! How old would stemline apogastropods, gastropods or molluscs have to be then? Authors or any other experts involved in the review process should have doubted such results, could have explored the reasons for this, and could have required giving

confidence intervals in an improved approach. The resulting reinterpretation of fossils and discussion of evolutionary scenarios in Medina et al. (2011) depends on unreliable topologies and highly unlikely time scales.

Concluding, both 'Opisthobranchia' and 'Pulmonata' were biased towards monophyly by definition and by selection. The problematic study design by Medina et al. (2011) clearly weakens the significance of topological results used for proposing a reclassification of 'Opisthobranchia', a taxon that is contradicted by almost all other recent studies (see review by Schrödl et al., 2011). Neither molecular timing, nor evolutionary conclusions by Medina et al. (2011) are convincing, and alternative interpretations must be considered. While generating an impressive amount of novel data, 'Crawling through time' has highlighted how slow progress can be in understanding sea slug evolution.

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M. Schrödl*
K.M. Jörger
Bavarian State Collection of Zoology, Münchhausenstrasse 21,
81247 München, Germany
* Corresponding author.
Michael.Schroedl@zsm.mwn.de (M. Schrödl).

N.G. Wilson
The Australian Museum, 6 College Street, Sydney 2010 NSW, Australia

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