Dating the Terrestrial Invasion of the Cyclophoroidea (Mollusca: Gastropoda) Using the Fossilized Birth-Death Model

Timothy Gervascio

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Dating the terrestrial invasion of the Cyclophoroidea (Mollusca:Gastropoda) using the Fossilized Birth-Death model.

By

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Abstract

Despite their vast diversity, few metazoan lineages have made the transition from marine to terrestrial habitats. These transitions must have been accompanied by dramatic shifts in the physiology and ecology of the lineages, marking major evolutionary events that have shaped the diversity we see today. Among the Metazoa, gastropod molluscs have been among the most successful at making the transition, comprising at least 12 separate transitions. This unusual history makes them particularly suitable for studying the evolutionary context of these major events. The development of new relaxed molecular clock methods for seamlessly integrating molecular and fossil data provides new possibilities for determining the timing of evolutionary events. Here we use the Fossilized Birth-Birth Death process implemented in BEAST v 2.4 to estimate the divergence time of the uniformly terrestrial Cyclophoroidea (Mollusca: Caenogastropoda) from the rest of the Caenogastropoda. Our analysis shows that the terrestrialization of the Cyclophoroidea occurred around the end of the Paleozoic, and may have been associated with the End Permian mass extinction. This places the Cyclophoroidea as the first caenogastropod clade to make the transition to terrestriality.
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Acknowledgements

I thank Dr. Rundell for her endless generosity and support as my advisor and teacher; and Jesse Czekanski-Moir for his help and patience as I worked on this project.

I would also like to thank David Bullis, Teresa Rose Osborne, and Alyssa Lau for their role in developing the larger questions that this research addresses.

And I would like to thank the Rundell Lab team as a whole for their enthusiasm for science and for their extraordinary senses of humor. I could not have imagined such a phenomenal group before I joined the lab.
Introduction

Transitions from marine to terrestrial habitats have been exceedingly rare among the Metazoa. These transitions must have necessitated novel physiological strategies, as requirements for osmoregulation, gas exchange, and thermoregulation are vastly different on land (Selden & Edwards, 1989; Selden, 2005). Such shifts in the biology of the colonizers have undoubtedly left a signature in the biodiversity we see today.

Additionally, the first introductions of new lineages and functions to terrestrial ecosystems fundamentally altered the geochemical characteristics of the earth’s surface and augmented the possibilities for future biotic interaction (Selden & Edwards, 1989; Kenrick et al., 2012). However rare, these transitions clearly mark major events in the history of the Metazoa that have shaped the geographic and biological template for evolution to act on, resulting in the particular range of terrestrial biodiversity we see today.

The earliest evidence of terrestrial metazoans comes from Ordovician paleosols with *Skolithos* trace fossils attributed to an ancestor of modern myriopods (Selden, 2005). Millipedes were followed by arachnid mites and the first insects by the early Devonian (Selden, 2005). A major molecular clock analysis of the Ecdysozoa has proposed somewhat older ages for these transitions than the fossil record alone, but supports the sequence of the fossil record (Rota-Stabelli et al., 2013). Among the Metazoa, gastropod molluscs have been the most numerous colonizers of land, comprising at least 12 separate transitions (Vermeij & Dudley, 2000; Kameda & Kato, 2011; Romero et al., 2016). This makes them a key group for studying the evolutionary context and repercussions of terrestrialization.
Terrestrialization has taken place throughout the Gastropoda, with the Stylommatophora containing the most species rich terrestrial clades. Some of the earliest terrestrial gastropod fossils from the Carboniferous were originally attributed to the Stylommatophora, but their placement has been in doubted (Bandel, 1993). Additionally, the age of the Stylommatophora has been estimated using molecular data to be somewhere in the late Mesozoic, making their terrestrial constituents substantially younger than previously thought (Dinapoli & Klussmann-Kolb, 2010).

The Caenogastropoda is the most species rich group of gastropods and its species employ diverse ecological strategies and inhabit many different habitats, including multiple independent transitions to terrestriality (Colgan et al., 2007). In contrast to the Stylommatophora, the history of the Caenogastropoda reaches back at least to the Carboniferous, where limpet type gastropods with convincing caenogastropod larval characteristics are known from the Mississippian (358-323 mya) Tamworth Belt rocks (Cook et al., 2008).

The Cyclophoroidea are a rich and highly endemic superfamily of terrestrial caenogastropods distributed throughout Southeast Asia, the Indo-Pacific, and South America. Fossil cyclophoroideans are known mainly from the Jurassic-Cretaceous Purbeck Group of Europe, but some less certain Paleozoic specimens have been assigned as well (Bandel, 1993). Convincing specimens have also come from Burmese amber (J. Czekanski-Moir, personal communication, Feb. 2017). This, combined with their basal placement in the Caenogastropoda and age of closely related groups (such as the Carboniferous Cerithioidea fauna), suggests their history is substantially deeper than the late Mesozoic (Bandel, 1993; Colgan et al., 2007).
Studying evolutionary events in the context of the history of life depends upon being able to reliably estimate their timing. This is often done in a phylogenetic context, using assumptions based on the clock-like accumulation of mutations in all genomes. By applying a substitution rate (substitutions/time) model to the phylogenetic tree (the branch lengths), it is theoretically possible to determine the timing of events on the tree. However, the nucleotide sequence alone does not contain any information about the timing of substitutions, so external information about the timing of the evolutionary process is required to calibrate the branch lengths to absolute time. Because it is impractical to experimentally determine the mutation rate for every loci and species on a given phylogenetic tree, other external information is required. Fossils are the most common type of external information because they contain phylogenetic information about the evolutionary process and can be dated to absolute time. By placing a fossil with a known age in the tree, the substitution rate model can be calibrated to absolute time and the timing of evolutionary events can be estimated. The development of new relaxed molecular clock methods allow for substitution rates to vary realistically across the tree, and new methods of integrating the fossil data into the tree provide the most realistic divergence time estimates available.

The Fossilized Birth-Death process (FBD) is a recently developed Bayesian method for inferring time-calibrated phylogenies (Stadler 2010; Heath et al., 2014). In contrast to node-dating methods, FBD treats the fossils and occurrence times as tips that were produced by the same evolutionary process as the extant taxa. The model describes the probability of a time tree and fossil placements based on the diversification rate,
turnover, and probability of sampling a fossil. This allows for the possibility that the fossils are extinct sister-taxa on branches of the extant tree, or that fossils are sampled ancestors of the extant taxa rather than an arbitrary minimum node age.

The most complete higher taxonomic level phylogenetic hypothesis for the Caenogastropoda to date places the Cyclophoroidea among completely marine and freshwater groups (Campanilidae, Ampullariidae) (Colgan et al., 2007). Additionally, recent work has proposed the monophyly of the Cyclophoroidea, suggesting a single ancestral terrestrialization event some time in their history (Webster et al., 2012). Here, we date the divergence of the Cyclophoroidea from the rest of the Caenogastropoda using seven fossils for calibration, including two new Cyclophoroidea taxa from Burmese amber.

Methods

Taxon Sampling

Twenty-six taxa were selected to cover the Cyclophoroidea, Ampullarioidea, Campaniloidea, Cerithioidea, Conoidea, and Viviparoidea (Table 1). Taxa, as well as publicly available sequence data for taxa within the Cyclophoroidea and for Conus miles were drawn primarily from Webster and coworkers’ study of cyclophoroidean phylogenetics and shell evolution (2012). Taxa from additional caenogastropod families, Ampullariidae and Viviparidae, were included to facilitate the placement of additional fossils. Two ampullariid species, Pila polita and Pomacea bridgesii, were selected to cover the maximum distance within the family (Hayes et al., 2009). Two species were selected from the Viviparidae by the same criteria (Richter, 2015). Campanile
symbolicum was selected as a potential sister taxa to the Cyclophoroidea based on previous phylogenetic work covering the whole of Caenogastropoda (Colgan et al., 2006). Planaxis sulcatus was selected for the same purpose based on BLASTN searches of the NCBI nucleotide database.

Fossils

Five fossils within the Cyclophoroidea and two fossils from other groups were used to calibrate branch lengths to absolute time (Table 1). Two fossils from Burmese amber (100mya) had more certain taxonomic placement, and were constrained to the Cyclophoridae and the Diplommatinidae respectively (for discussion of constraints, see below) (J. Czekanski-Moir, personal communication, Feb. 2017). Three additional fossils from the European Purbeck Group (late Jurassic, early Cretaceous), while certainly members of the Cyclophoroidea, had a less explicit position within the Cyclophoroidea (Bandel, 1993). These three fossils were positioned within the Cyclophoroidea by the FBD model.

One fossil, Viviparus langtonensis (170 mya) was assigned to the Viviparidae (Richter, 2015; Tracey et al., 1993). One fossil, Ampullaria nipponica (160 mya) was assigned to the Ampulariidae (Tracey et al., 1993).

Molecular Sequence Matrix

Publicly available sequence data for five loci was collected from GenBank (Table 2). Eukaryotic SSU and LSU rRNA sequences were aligned using the SILVA rRNA database, which maintains highly curated and quality controlled rRNA alignments (Quast et al., 2013). All other alignments were carried out using ClustalW as implemented in
MEGA v 7.0 (Kumar et al., 2016). 16s rRNA sequences were aligned as non-coding nucleotides, cytochrome oxidase 1 (CO1) sequences were aligned as coding nucleotides using the invertebrate mitochondrial code, and histone H3 (H3) was aligned as coding nucleotides with the standard genetic code. We identified the best fit substitution model using AIC calculated by the “Find Best Models” tool in MEGA 7. 16s, 28s, CO1, and H3 were best fit by GTR + Γ and 18s was best fit by SYM.

The Fossilized Birth-Death Process and Phylogenetic Inference

Here, we use the Fossilized Birth-Death model with Sampled Ancestors implemented in BEAST v 2.4 (Drummond et al., 2012). In this method, fossils are assigned to a user defined clade based on prior information rather than to a node, and their placement in the topology of the clade is subject to the model parameters. Table 2 describes our user defined fossil placements. Substitution models used for phylogenetic inference are described above. Each gene was assigned an independent relaxed log normal molecular clock, meaning the substitution rate of a gene was drawn from a log normal distribution independently for each branch. A linked tree topology was inferred by BEAST using information from all five loci. We assigned 350mya as the origin time (x₀) of the branching process based on the earliest definitively caenogastropod fossils (Cook et al., 2008). The “Belau clade” of the Diplommatinidae, including Hungerfordia and Palaina, was constrained to be no older than 30mya by assigning a bounded uniform prior to the node to reflect the geological age of the archipelago they are endemic to (Crombie & Pregill, 1999; Rundell, 2008).

Posterior probabilities were estimated from three MCMC chains run for $2 \times 10^8$ generations, sampling every 1000 generations on the CIPRES Science Gateway (Miller et
Convergence was assessed by viewing marginal probability distributions, and mixing was assessed by viewing time series plots in Tracer v 1.6 (Rambaut et al., 2014). The MCMC chain was searched for the maximum clade credibility tree using LogCombiner and TreeAnnotator with a burn-in of 200 trees (Drummond et al., 2012).

Results

The maximum clade credibility tree from these FBD analyses produced a mid-Carboniferous to mid-Permian age range for the divergence of the Cyclophoroidea from the other caenogastropods included in the analyses (Figure 1). All other Caenogastropoda included were more closely related to each other than to the Cyclophoroidea. The analyses revealed a Permian – Triassic age range for the crown Cyclophoroidea and a Jurassic age for the two largest clades within Cyclophoroidea, the Diplommatininae (Diplommatinidae minus Cochlostomatinae) and the Cyclophoridae (Figure 1).

Discussion

The time calibrated phylogenetic hypothesis based on 26 extant taxa and 7 fossil calibration points revealed a mid-Carboniferous to mid-Permian age for the divergence of the Cyclophoroidea from the rest of the Caenogastropoda and a Permian-Triassic age for the uniformly terrestrial crown Cyclophoroidea, suggesting the transition to terrestriality may have been associated with the End-Permian mass extinction.

The End-Permian mass extinction was the most extreme of earth’s five mass extinctions, resulting in the sudden loss of 90% of marine species and a significant turnover of terrestrial life. The timing of the Permian-Triassic (PT) boundary coincides
with a massive introduction of CO₂ and methane, a global drop in O₂, and a spike in atmospheric sulfates in the geologic record attributed to volcanism, declining sea level, and a general breakdown of the marine carbon cycle (Jin et al., 2000; Shen et al., 2011). In addition to the sudden turnover of marine species, terrestrial environments were marked by a collapse of tropical *Gigantopteris* flora and global wildfires as a result of climate change (Shen et al., 2011). Marine conditions were characterized by regional sea level declines, a corresponding increase in salinity, and anoxia in deep waters beyond the reach of mixing (Erwin et al., 2002; Knoll et al., 2007).

Changes in marine conditions coinciding with the proposed timing of Cyclophoroidea terrestrialization offer important information about selective pressures that may have factored into terrestrialization. Knoll and coworkers (2007) suggest that shallow waters could have acted as biological refugia during the PT event as deeper waters became anoxic due to a breakdown in mixing or to climatic factors. The survival of the ancestral Cyclophoroidea in those conditions could have been contingent upon making the transition to shallower waters (i.e. closer to land) or even to terrestrialization itself.

While the described analyses can provide context for the evolutionary shift from marine to terrestrial habitats in the Cyclophoroidea, some questions still remain. The divergence of the ancestrally marine Cyclophoroidea from the rest of the Caenogastropoda happened earlier than the events surrounding the PT extinctions. If the divergence of the Cyclophoroidea was a result of terrestrialization (before the PT events) the uniform terrestriality of extant Cyclophoroidea could be understood as contingent upon the terrestrial Cyclophoroidea avoiding the worst of the PT events.
Another interpretation is to view the crown age of extant Cyclophoroidea as a result of a radiation following terrestrialization. This hypothesis is more in line with the immediate PT mechanisms discussed above as the 95% CI is centered almost exactly on the PT boundary (256.8 mya). This could also indicate that terrestrialization occurred sometime through the Triassic as ecosystems recovered and previously occupied niches were opened. Unfortunately, we lack a full understanding of how recovery occurred following the PT extinction due to the depauperate early Triassic fossil record (Chen & Benton, 2012).

In comparison to other independent gastropod terrestrialization events, the Cyclophoroidea were likely one of the first to make the transition. Members of the Truncatellidae, Assimineidae, and Pomatiopsidae made the transition in the Cenozoic, while the Helicinae, Hydrocenidae made the transition in the Mesozoic (Kameda & Kato, 2011; Kano et al., 2002). Carboniferous fossils previously thought to be terrestrial stylommatophorans are in doubt, and recent molecular dating of the Heterobranchia suggests a much later Mesozoic diversification and terrestrialization (Bandel, 1993; Dinapoli & Klussmann-Kolb, 2010). Only the extinct Dawsonellidae is known from the Paleozoic (Kano et al., 2002). This leaves the possibility that the Cyclophoroidea have the longest history on land among the Gastropoda, and confirms that they have the longest history on land of any caenogastropod clade.

The ecology of these extant lineages suggests the timing and context of terrestrialization matter greatly in determining the trajectory of the lineage. The modern Cyclophoroidea mainly feed on lichens and detritus, while stylommatophorans are more often specialized herbivores. The Stylommatophora also appear to have transitioned to
land during the late Mesozoic as angiosperms radiated, while the Cyclophoroidea predate the radiation of flowering plants. In the case of the Cyclophoroidea, this is likely the ancestral dietary state, suggesting significant conservation in their ecology despite comprising substantial species diversity. This may be characteristic of terrestrial gastropods as a whole, where modern examples of nonecological speciation are common and low vagility makes geography a potent barrier to gene flow (Rundell & Price, 2009; Rundell, 2011). Our data extends this observation into the realm of macroevolution.

Estimating of the timing of major evolutionary events is an important part of understanding the history of life on earth. Despite the limited communication between neontology and paleontology, synthesis is a critical goal for modern evolutionary biology (Grantham, 2004). Time calibrated phylogenies are a common source of interaction between the fields, and are only improved by the recent explosion of publicly available molecular data and the development of new methods for applying paleontological data to molecular based trees.

**Conclusion**

The transition from marine to terrestrial habitats marks a major event in the evolution of a lineage, necessitating novel evolutionary and ecological trajectories that shape the diversity of life we see today. Gastropod molluses have been among the most successful metazoans crossing this seemingly impassable physical boundary, making gastropods important systems for studying the evolutionary context of terrestrialization events. Our analyses place the divergence of the completely terrestrial Cyclophoroidea from the other Caenogastropoda in the mid-Carboniferous to mid-Permian preceding the PT mass extinction. The crown age of the Cyclophoroidea falls directly over the PT
boundary, suggesting the largest mass extinction in Earth’s history may have played a
role in the transition. The terrestrialization of the Cyclophoroidea is likely the oldest
among the caenogastropods and one of the earliest in all of Gastropoda. As the amount of
available molecular and fossil data continues to swell, time calibrated phylogenies like
the presented here are an important source of interaction between the historically isolated
fields of neontology and paleontology.
References


Table 1. Seven fossils used to calibrate the phylogeny of extant Cyclophoroidea. In contrast to node-dating methods, the Fossilized Birth-Death model assigns fossils to a monophyletic clade and allows them to be positioned as a stem taxa to the clade or as a sampled ancestor of an extant taxa. The placement of fossils is referred to in the “FBD Clade Assignment” column.

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<th>Species</th>
<th>FBD Clade Assignment</th>
<th>Age (mya)</th>
<th>Source</th>
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### Table 2. Extant taxa and associated GenBank accession numbers used to generate the phylogeny of the Cyclophoroidea.

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<td>Viviparidae</td>
<td><em>Bellamy jeffrey</em></td>
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<td>FJ405649</td>
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<td><em>Angulyagra sp. J AJ 2009</em></td>
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Figure 1. The maximum clade credibility tree of the Cyclophoroidea based on five loci (16s, 18s, 28s, COI, H3). Time calibration was carried out using the Fossilized Birth-Death process and seven fossil calibration points. Node labels indicate the age of the node in billions of years and node bars indicate 95% CI. The Cyclophoroidea diverged from the rest of the Caenogastropoda in the mid Carboniferous to mid Permian. The crown Cyclophoroidea are Permain to Triassic in age, centered close to the End Permian mass extinction.