Molecular Phylogenetics and Evolution 97 (2016) 43–54

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogeny of the Ellobiidae (Gastropoda: Panpulmonata) supports independent terrestrial invasions ☆

Pedro E. Romero [a,b,*], Markus Pfenninger [a,c], Yasunori Kano [d], Annette Klussmann-Kolb [e]

[a] *Senckenberg Biodiversity and Climate Research Centre (BiK-F), Senckenberg Gesellschaft für Naturforschung (SGN), Senckenberganlage 25, Frankfurt am Main 60325, Germany*
[b] *Institute for Ecology, Evolution & Diversity, Goethe University, Max-von-Laue-Straße 13, Frankfurt am Main 60438, Germany*
[c] *Institute for Ecology, Evolution & Diversity, Goethe University, Max-von-Laue-Straße 13, Frankfurt am Main 60439, Germany*
[d] *Atmosphere and Ocean Research Institute, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan*
[e] *Zoologisches Forschungsmuseum König, Adenauerallee 160, 53113 Bonn, Germany*

ARTICLE INFO

Article history:
Received 22 April 2015
Revised 20 November 2015
Accepted 21 December 2015
Available online 24 December 2015

Keywords:
Carychiinae
Pythiinae
Terrestrial invasion
Realm transitions
Molecular clock
Topology tests

ABSTRACT

Gastropods of the family Ellobiidae are an interesting group in which to study transitions from intertidal to terrestrial realms. However, the phylogenetic relationships within this family still lack resolution. We present a phylogenetic hypothesis of the Ellobiidae based on Bayesian and maximum likelihood phylograms. We used nuclear (18S, 28S, H3) and mitochondrial (16S, 12S, COI) data, increasing the numbers of markers and data, and making this the most comprehensive phylogenetic study of the family to date. Our results support phylogenetic hypotheses derived from morphological data, and provide a supported framework to evaluate the internal relationships within Ellobiidae. The resulting phylogenetic trees support the previous hypothesis that the Ellobiidae are monophyletic only if the Trimusculinae (*Otina*, *Smeagol* and *Trimusculus*) are considered part of this family. In addition, we found that the Carychiinae, Ellobiinae and Pythiinae are reciprocally monophyletic and closely related, with the Carychiinae as sister group to Ellobiinae. Relationships within Melampodinae and Pedipedinae and their phylogenetic positions remain unresolved. Land invasion by the Ellobiidae occurred independently in Carychiinae and *Pythia* during different geological times (Mesozoic and Cenozoic, respectively). Diversification in the family does not appear to be related to past climate and biotic changes, neither the Cretaceous–Paleogene boundary nor the lowering of the sea level in the Oligocene.

1. Introduction

The conquest of land by organisms that evolved from aquatic ancestors represents one of the most astonishing events in Earth's history of life (Lillywhite, 2012). This step was achieved multiple times in different phyla by means of specific adaptations in osmoregulation and water balance, air breathing, nitrogen excretion, reproduction, locomotion and behavior (Little, 1990). Gastropods are one of the most successful taxa to have developed terrestrial lineages; land invasion has been achieved several times, for example in Neritimorpha, Caenogastropda, and Heterobranchia (Kameda and Kato, 2011).

Within Heterobranchia, the clade Panpulmonata includes several lineages that invaded the intertidal zone and non-marine ecosystems, for instance, Acochlidia, Ellobiidae, Hygrophila, Stylommatophora and Systellommatophora (Klussmann-Kolb et al., 2008; Jörger et al., 2010; Kano et al., 2015). Previous phylogenetic studies suggested that the evolution of panpulmonate terrestriality has occurred multiple times (Barker, 2001), although there is still controversy about the direction of the transitions between realms. Only solid phylogenetic hypotheses can serve as a framework to clarify the origins of this ecological diversity and the habitat transitions. Panpulmonate phylogeny therefore has important implications for understanding how gastropods transitioned out of marine habitats, resulting in one of the most significant adaptive radiations among animals (Kocot et al., 2013).

The panpulmonate family Ellobiidae (hollow-shelled snails) are a characteristic component of the intertidal and supratidal zones of mangrove forests and muddy shores in tropical regions worldwide, as well as of salt marshes and upper littoral rocky areas in temperate regions (Martins, 1996). In addition, three ellobiid genera include truly terrestrial species, which are independent from any

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☆ This paper was edited by the Associate Editor Jan Strugnell.

* Corresponding author at: Senckenberg Biodiversity and Climate Research Centre (BiK-F), Senckenberg Gesellschaft für Naturforschung (SGN), Senckenberganlage 25, Frankfurt am Main 60325, Germany.

E-mail addresses: pedro.romero@senckenberg.de, quipu.romero@gmail.com (P.E. Romero), pfenninger@bio.uni-frankfurt.de (M. Pfenninger), kano@aori.u-tokyo.ac.jp (Y. Kano), a.klussmann-kolb@zfmk.de (A. Klussmann-Kolb).

http://dx.doi.org/10.1016/j.ympev.2015.12.014
1055-7903/

aquatic habitats: *Pythia* Röding, 1798 in the Indo-western Pacific (for instance *Pythia colmani* Martins, 1995 living in the rainforest of Papua New Guinea; Martins, 1995), *Carychium* Müller, 1773 in the litterfall of forests and Holartic riparian zones, and *Zospeum* Bourguignat, 1856 in European karst caves (Barker, 2001). Approximately 800 species names have been proposed for ellobiids, of which 100 (Mordan and Wade, 2008) to 250 (Weigand et al., 2013) are likely to be valid. The latest classification proposes five subfamilies within the Ellobiidae: Ellobiinae, Carychiinae, Melampodinae, Pedipedinae, and Pythiinae (Martins, 2007), although some authors have recognized a family rank for Carychiinae (Jörger et al., 2010; Weigand et al., 2013).

Morphological data have not been sufficient in determining the phylogenetic relationships within Ellobiidae, nor the systematic position of the family among panpulmonates (Morton, 1955; Dayrat and Tillier, 2002; Martins, 2007). However, the subfamilies can be distinguished by anatomical characters in the reproductive and nervous systems. The Carychiinae, Pedipedinae and Pythiinae are monoaulic (i.e. they possess a single hermaphroditic duct), whereas the Ellobiinae are diaulic (male and female ducts run separately), and the Melampodinae are semidiaulic (male and female gonoducts distally separated). In addition, the Pedipedinae and Melampodinae can be distinguished from species in the other subfamilies by their short visceral nerve ring (Martins, 2007).

Previous molecular phylogenetic studies were also not able to resolve neither the systematic position of the Ellobiidae nor the relations of the clades within. Complete mitochondrial genomes of ellobiids have been used in previous studies aiming to reconstruct the phylogeny of Gastropoda (Grande et al., 2008) or Heterobranchia (Medina et al., 2011; White et al., 2011). However, these phylogenetic analyses based only on mitochondrial data received criticism (Stöger and Schrödl, 2013; Wägele et al., 2014) and contradicted the results from multi-locus (nuclear and mitochondrial) analyses (Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010) and phylogenomic studies (Kocot et al., 2013; Zapata et al., 2014). Long branch attraction artifacts were assumed to infer an erroneous rooting in the mitochondrial trees (Schrödl et al., 2011; Schrödl, 2014). Multi-locus studies have used a core set of molecular markers, including the nuclear 18S rRNA and 28S rRNA, and mitochondrial 16S rRNA and cytochrome oxidase I (COI) (Klussmann-Kolb et al., 2008; Jörger et al., 2010; Dayrat et al., 2011). In addition, it has been hypothesized that the family includes the genera *Otina* Gray, 1847 and *Smeagol* Climo, 1980 (formerly Otinidae), and *Trimusculus* Schmidt, 1818 (Trimusculidae), also occurring in the intertidal zone (Dayrat et al., 2011). However, relationships among the subfamilies have remained unclear, making it difficult to interpret the time and occurrence of habitat transitions.

In this work, we reconstructed a phylogenetic hypothesis of the Ellobiidae with increased numbers of taxa (comprising all subfamilies) and loci (nuclear 18S rRNA, 28S rRNA and Histone H3, and mitochondrial 16S rRNA, 12S rRNA and COI). Our topology showed improved phylogenetic resolution within Ellobiidae, allowing us to evaluate the monophyly of the subfamilies and to test the current hypotheses of the tempo and mode of habitat transitions. We found that ellobiids colonized the land independently in Carychiinae and Pythiinae during the Mesozoic and Cenozoic periods, respectively.

2. Material and methods

2.1. Taxon sampling

A total of 63 ellobiid species comprising 23 genera were included in this study. We followed the suggestions by Martins (2007) for the nomenclature of the subfamilies. Recent studies using a multilocus (Jörger et al., 2010; Dayrat et al., 2011) or phylogenomic approach (Zapata et al., 2014) have shown that the Systellommatophora are the sister group of the Ellobiidae. Thus, the outgroup included 11 species from Onchidiidae (Systellommatophora). Specimens of all five ellobiid subfamilies were included in our data matrix: Carychiinae (2 genera used/2 genera accepted in Martins (2007)), Ellobiinae (5/5) Melampodinae (3/5), Pedipedinae (2/4), and Pythiinae (8/8). Data sets from previous studies (Klussmann-Kolb et al., 2008; Dinapoli and Klussmann-Kolb, 2010; Dayrat et al., 2011; Vonnemann et al., 2005; Weigand et al., 2011, 2013) served as a starting point for our analyses, in particular sequences of 18S rRNA, 16S rRNA and COI genes. The matrix was expanded with three additional markers: nuclear 28S rRNA and Histone H3, and mitochondrial 12S rRNA. In addition, taxon sampling was extended, e.g. 20 of the 63 ellobiid species now included were previously absent in GenBank. The mean ratio of sequences per marker was 81%, ranging from 100% for 18S rRNA to 64% for H3. Samples are preserved in the collection of the Senckenberg Naturmuseum Frankfurt; detailed information is shown in Table 1. We also considered the genera *Otina*, *Smeagol* and *Trimusculus* as members of the Ellobiidae. The close relationships of these genera to the above five subfamilies were previously proposed by Klussmann-Kolb et al. (2008) and Dinapoli and Klussmann-Kolb (2010), and then confirmed by Dayrat et al. (2011) using a wider taxonomic sampling.

Ellobiids exhibit modest diversity on land (Barker, 2001). In our analysis, terrestrial genera were represented in Carychiinae (*Carychium* and *Zospeum*) and Pythiinae (*Pythia* sp.). The latter species of *Pythia* was collected from a tropical rainforest in the Republic of Palau, where associated mollusks were all typical land snails of Cyclophoroidea and Stylommatophora, and was verified as being terrestrial throughout its ontogeny (Kano et al., 2015). Other species of the genus living on mangrove trees, such as *Pythia scarabaeus* (Linnaeus, 1758), were considered as intertidal. Members of Carychiinae were also considered fully terrestrial because of their geographic and ecological distributions (forests or caves that are far from the sea).

2.2. DNA extraction, amplification and sequencing

Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Germany) from specimens stored in 100% ethanol. A small piece ($\leqslant 2$ mm^3) of the foot was used, and the shell was destructed in case of very small samples. Polymerase chain reactions (PCR) were performed to amplify fragments from the six markers mentioned above; Supplementary Table S1 shows primers used in the PCR reactions. The reactions followed this protocol: Each 25 µL PCR mixture included 1 µL (10 pmol) of each primer, 2.5 µL 10× PCR buffer, 2 µL (100 mM) $MgCl_2$, 0.2 µL (20 mM) dNTPs, 0.3 µL Taq-polymerase (Fermentas), 1.5 µL (10 mg/mL) bovine serum albumin, 12.5 µL ddH_2O and 4 µL template DNA. PCR cycles were done with these conditions: 1 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 52 °C and 30 s at 72 °C, and finally, 3 min at 72 °C. Visualization of PCR products was performed on a 1.4% agarose gel. Amplicons were cleaned using the QIAquick PCR Purification Kit or the QIAquick Gel Extraction kit (Qiagen) whenever multiple bands were detected. PCR products were bidirectionally sequenced using the PCR primer pair (5 pmol) and the BigDye® Terminator v.3.1 Cycle Sequencing Kit (LifeTechnologies, Inc.) on an ABI 3730 capillary sequencer following the manufacturer's instructions. The sequencing reactions were performed in the BiK-F Laboratory Centre, Frankfurt am Main. Sequences were deposited in GenBank with the following accession numbers: 18S: KM280960–KM281008, 28S: KM281048–KM281088, 16S: KM281010–KM281044, 12S: KJ920288–KJ920333, COI: KM281099–KM281117, H3: KM281118–KM281155.