

# Dating of the Limnadiidae family suggests an American origin of *Eulimnadia*

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**Abstract** The spinicaudatan clam shrimp is a group of branchiopod crustaceans that has existed as far back as the Devonian and well-preserved fossils are known. Evidence of monophyly exists for only one family (Limnadiidae), which has a worldwide distribution and morphological conservatism. The evolutionary relationships among genera and diversification mechanisms are not deeply resolved as well as origin of the genus *Eulimnadia*. To address these issues, we constructed phylogenies of limnadiid clam shrimp, with both Bayesian inference and maximum likelihood methods to infer limnadiid evolutionary relationships. We then performed dated phylogenies using a relaxed clock of the Spinicaudata using fossil calibrations. Divergence date estimates show a perfect match with the break up of the Pangaea that could explain current limnadiid distributions; however the genus *Eulimnadia* apparently diverged 30 Ma ago.

*Eulimnadia* phylogeography suggests an American origin and ecological patterns were analyzed to propose hypotheses on its origin and spread. This genus also shows a strong dispersive capacity, which could be explained by its reproduction modalities (androdioecy). This study and this first phylogeny with fossil calibration date the current distribution of Spinicaudata and reveal congruence with continental drift, except for *Eulimnadia*.

**Keywords** Androdioecy · Divergence time estimation · Fossil calibration · Limnadiidae · Phylogeography · Spinicaudata

## Introduction

Spinicaudata is a suborder of branchiopod crustaceans (4–30 mm long) with a folded carapace and laterally compressed bodies. These continental organisms are typically found in temporary pond ecosystems in various climates. To survive in this type of biotope, especially during the dry season, many branchiopods produce resting eggs (Brendonck et al., 2008; Rogers, 2014). In addition, resting eggs disperse passively, which allows the colonization of new ponds. The taxonomy of this group and the understanding of its evolution are still controversial and have recently been challenged by molecular studies (Hoeh et al., 2006; Weeks et al., 2009). Only three families are currently

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recognized: Limnadiidae (Burmeister, 1843), Leptotheriidae, and Cyzicidae (Stebbing, 1902) but monophyly is supported only for the Limnadiidae (Hoeh et al., 2006). Nine genera belong to the Limnadiidae: *Australimnadia*, *Calalimnadia*, *Eulimnadia*, *Gondwanalimnadia*, *Imnadia*, *Limnadia*, *Limnadopsis*, *Metalimnadia*, and *Paralimnadia* (Rogers et al., 2012; Timms & Schwentner, 2012; Rogers et al., 2016). The limnadiids are known to be variable in their reproduction modes: while most genera are gonochoric, others are hermaphroditic with rare males (*Limnadia lenticularis*) or without males (*Calalimnadia mahei*). In *Eulimnadia*, androdioecy (populations comprised a mixture of males and hermaphrodites) seems to be the general rule even if some populations are only composed of hermaphrodites. It is also interesting to note that the limnadiids show a worldwide distribution (excluding Antarctica). *Limnadia* and *Eulimnadia* both have a particularly vast distribution area as opposed to all other limnadiids genera. These different distributions are not yet understood and, until now, the only assumption justifying the large distribution of *Eulimnadia* is the hypothetical old age of this genus (Weeks et al., 2006).

One way to understand the global distribution of limnadiids is to construct a complete phylogeny with all the lineages and infer divergence time among genera. The Spinicaudata, including the Limnadiidae, and their extensive fossil record are known from the Devonian (Tasch, 1967; Novojilov, 1970; Astrop & Hegna, 2015; Gueriau et al., 2016).

Over the past 50 years, independent groups have comprehensively reviewed spinicaudatan phylogenetics (Astrop & Hegna, 2015). To our knowledge, there is no phylogenetic study using fossil calibrations to deduce the age of different Limnadiidae lineages.

The aim of this work is to explain the global distribution and evolution of the Limnadiidae. The first step was to perform a 28S ribosomal DNA phylogeny of the Limnadiidae. Then, we investigated spinicaudatan divergence times with fossil calibrations. Finally, we conducted a phylogeography of *Eulimnadia*. In order to explain the distribution of *Eulimnadia* we examined ecological characteristics, such as life cycle diversity and experimental breeding.

## Materials and methods

### Sample

A unique sample of the limnadiid sp. 1 from Bolivia was collected and substrate samples from limnadiid habitats were collected following Rabet et al. (2014), available locality data are presented in Table 1. To culture adults we placed 100 g of soil with resting eggs in 10 l milliQ water at 28°C with a permanent light. Development was checked twice daily. The adult stage was identified when resting eggs were visible under the carapace of females or hermaphrodites. The

**Table 1** Locality data on branchiopods used in this study

Taxon	Locality data
<i>Calalimnadia mahei</i> Rogers et al. 2012	REPUBLIC OF MAURITIUS: Cap Malheureux, La Mort temporary pool, 19 April 2001, N. Rabet
<i>Eulimnadia colombiensis</i> Roessler, 1990	BRAZIL: Bahia, temporary pool at Itapetinga, 22 November 2012, S. Lacau
<i>Eulimnadia cylindrova</i> Belk, 1989	FRANCE- MARTINIQUE: Saint Anne, Plage des Salines temporary pool, 30 July 2003, N. Rabet
<i>Eulimnadia magdalenensis</i> Roessler, 1990	BRAZIL: Bahia, sandy temporary pool at Barrolândia, 16 June 2008, S. Lacau and M. Pignal
<i>Imnadia yeyetta</i> Hertzog, 1935	FRANCE: Bouches du Rhône: Cerisière Nord, Tour du Vallat, November 2010, N. Rabet
<i>Limnadia lenticularis</i> L., 1761	FRANCE: Alsace: Munchausen, temporary pool, March 2014, N. Rabet
<i>Limnadiidae</i> lineage BO sp. 1	BOLIVIA: Cullucachi, ditch between Batallas and Laja near Pucarina, altitude of 3,860 m, 13 February 2007, M. Manuel
<i>Metalimnadia</i> sp.	BRAZIL: Paraíba, Esperança, dry soil, 28 July 1993, N. Rabet BRAZIL: Bahia, Palmas de Alto, 9 November 2008, M. Rodrigues da Silva

longevity was given following the maximum age reached by several individuals from the same cohort (in artificial conditions, death is regularly observed inside the cohort but some individuals seems to reach the natural limit of the species).

#### DNA extraction, PCR amplification, and sequencing

DNA extraction of the limnadiid sp. 1 from Bolivia was done following the Qiagen amplification DNA Mini Tissue Kit protocol from the whole specimen, stored since sampling in 96° ethanol. The 28S rRNA was amplified with the primer set D1D2fw1/D1D2rev2 (Sonnenberg et al., 2007). PCR reaction was performed with 2 µl of DNA extraction in a 20 µl final volume (0.32 µl of each primer at 10 µM, 0.8 µl of dNTP-mix at 6.6 mM, 1 µl of Bovine Serum Albumin at 1 mg/ml, 2 µl PCR buffer, and 0.12 unit Taq polymerase (Taq DNA polymerase, Qiagen). Cycler settings were conducted in a Mastercycler (Eppendorf) with an initial step of 94°C for 4 min, 45 cycles at 94°C for 20 s (denaturation), 52.5°C for 20 s (primer hybridization), 72°C for 90 s (elongation), and a final elongation at 72°C for 8 min. Successful PCRs were selected on ethidium–bromide-stained agarose gels. Sanger sequencing (both directions) was performed by a commercial company (Eurofins; <http://www.eurofins.fr>) using the same primers. Chromatograms in both directions were compared and a consensus sequence was assembled using Bioedit (Hall, 1999).

#### Phylogenetic reconstructions of Spinicaudata and *Eulimnadia*

An alignment of 28S ribosomal DNA sequences from 40 selected spinicaudatans (Online resource 1) was performed with Muscle (Edgar, 2004) using Geneious version 8 (<http://www.geneious.com>, Kearse et al., 2012). Gaps were removed using Gblocks (Castresana, 2000) carried out on the phylogeny.fr platform (Dereeper et al., 2008). Phylogenetic reconstructions were performed using both Bayesian inference (BI) and maximum likelihood (ML) from an alignment of 40 sequences of 748 bp. Evolutionary models were selected via Akaike Information Criterion or Bayesian Information Criterion using jModelTest v2.1.1 (Darriba et al., 2012). Maximum likelihood was performed

using PhyML (Guindon & Gascuel, 2003) under Geneious v8 with a general time reversible model with a gamma distribution ( $\Gamma = 0.754$ ) and a proportion of invariable sites ( $I = 0.578$ ), validated with 1,000 bootstrap replicates. Bayesian analyse were carried out and done with MrBayes 3.2.3 (Ronquist & Huelsenbeck, 2003) on the CIPRES Science Gateway (Miller et al., 2010), with 4 chains of  $1 \times 10^6$  generations, trees sampled every 100 generations, and burn-in value set to 20% of the sampled trees. Sequences were considered with an evolutionary model (TIM3) with a gamma distribution ( $\Gamma = 0.754$ ) and a proportion of invariable sites ( $I = 0.578$ ). We checked that standard deviation of the split frequencies fell below 0.01 and confirmed convergences of the runs to ensure convergence in tree search using the Tracer v1.6 software (<http://tree.bio.ed.ac.uk/software/tracer/>). Trees were visualized using FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

An alignment of 102 sequences (860 bp) of 28S ribosomal DNA identified as *Eulimnadia* in Genbank (all sequences available) was created. Protocol described above was used for the reconstruction of this *Eulimnadia* phylogeny. Maximum likelihood was performed with an evolutionary model (TIM2) plus a proportion of invariable sites ( $I = 0.88$ ), and validated with 1,000 bootstrap replicates. Bayesian analyse were carried out with 4 chains of  $2 \times 10^6$  generations, trees sampled every 200 generations, and burn-in value set to 20% of the sampled trees. Sequences were considered with an evolutionary model (TIM2) plus a proportion of invariable sites ( $I = 0.88$ ).

#### Divergence time estimation

We constructed two datasets: the first containing 42 taxa (36 representative species of Spinicaudata and 6 notostracan outgroups) to investigate global Limnadiids emergence dates. The second one corresponds to the 36 Spinicaudatan taxa used to investigate Limnadiidae evolutionary history. Alignments were performed with Muscle and gaps were removed with Gblocks as described above. BEAST software package v2.1.3 (Bouckaert et al., 2014) was used to infer divergence time. We applied the uncorrelated lognormal relaxed clock model (Drummond et al., 2006) to account for rate heterogeneity among lineages. This model was evaluated with the CoV, where CoV values  $>0$  were considered as evidence of non-clock

evolutionary behavior. The calibrated Yule process (Heled & Drummond, 2012), an extension of the birth–death model, was selected as tree prior for the model of speciation. We applied a GTR+I+G to the complete dataset and a TN 93 +I+G to the Spinicaudata dataset, referring to evolutionary model obtained by jModelTest v2.1.1 (Darriba et al., 2012). For calibrating the phylogenetic tree we used 2 branchiopod fossils (*Castracollis wilsonae* (Fayers & Trewin, 2002)) and *Afrolimnadia sibiriensis* (Tasch, 1987) plus an estimation of the split between the genera *Triops* and *Lepidurus* with normal prior distribution. The Pragian fossil *C. wilsonae* was chosen for inferring the minimum age of the last common ancestor of Notostracan and Spinicaudata (Fayers & Trewin, 2002) because it shares characters from the Notostraca lineage and the Diplostraca (including Spinicaudata) (Olesen, 2009; Lagebro et al., 2015). We used the lower boundary of the Pragian stage (407.6 ( $\pm 1.3$ ) Ma) (Cohen et al., 2013) with a normal distribution prior for this node. The Notostraca order split (*Triops* split from *Lepidurus*) was estimated at 188 Ma, inferred by recent publications on molecular phylogeny and divergence time of notostracan (Korn et al., 2013; Mathers et al., 2013).

We chose the Sinemurian fossil *A. sibiriensis* as calibration for the Limnadiidae age estimates with a normal distribution at 190.8 ( $\pm 0.5$ ) Ma. Indeed this species has an important space without growth lines and a fragile carapace, which suggests that this species is a Limnadiidae. The space without growth lines can be explained because during the early stages, the molting of the valves is complete; the animals do not retain the external laminae of previous stages. The older laminae remain, overlaying the younger and larger plates, and forming the well-known growth lines of the typical Spinicaudatan carapace (Roessler, 1995). Our choices for spinicaudatan fossils were made in light to the lack of compatibility between biological and paleontological studies in this group. For current species, the taxonomic status is based on molecular characters associated with morphological characters such as the head or the furca and sometimes the carapace may be informative also, but never essential. In paleontology, the shape of the carapace is essential because no other details are generally found; these insufficiencies of characters imply recurrent incompatibility with modern systematics but recently

a first assay of synthesis was initiated (Astrop & Hegna, 2015).

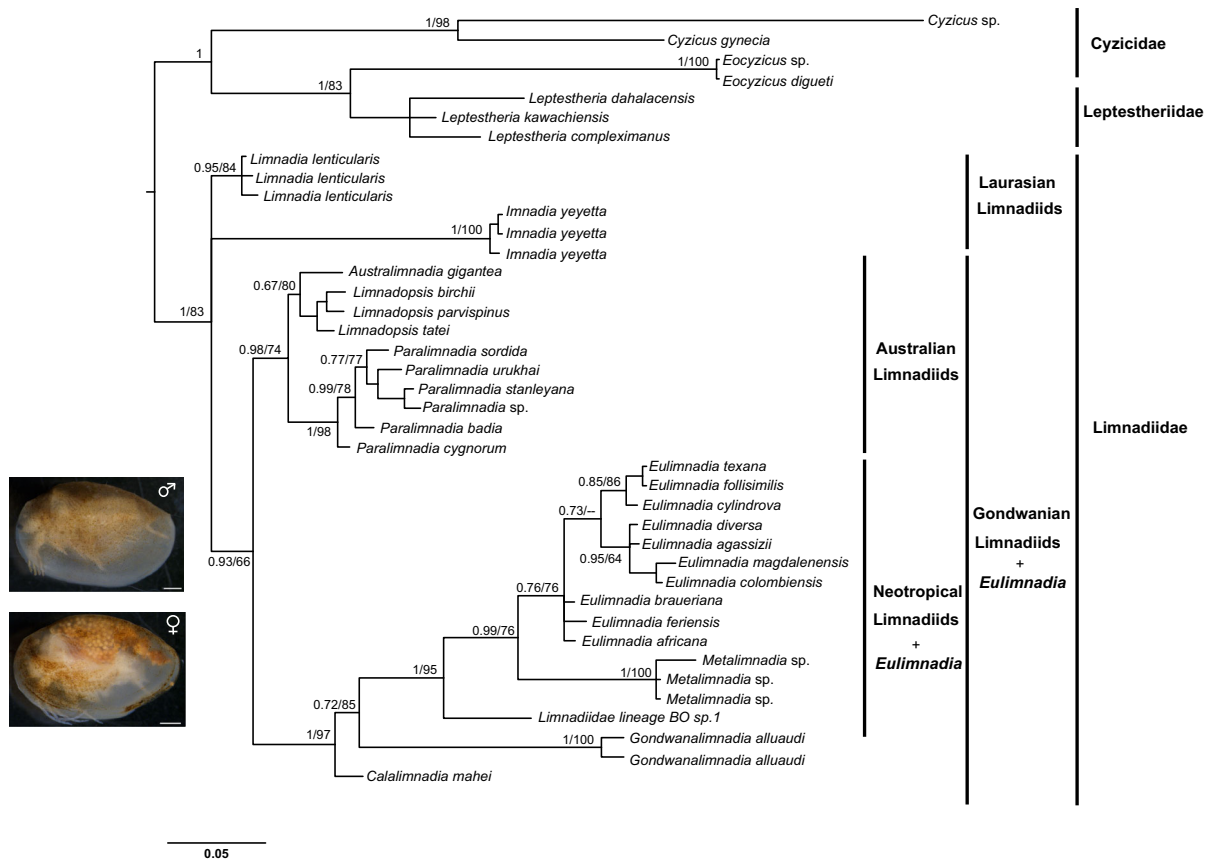
For the dataset with only Spinicaudata we used only one fossil calibration but two analyses were performed. For the first, we considered divergence times with the Sinemurian fossil but for the second we assumed a more conservative approach with a Perilimnadiidae fossil (Chen, 1975), which exhibits large carapace with limnadiform shape plus preservation of carapace gland (Zhang et al., 1976). This family was indicated to be sister group of the *Limnadiidae* within an attempt of clear phylogeny investigation between living and fossil spinicaudatan taxa (Astrop & Hegna, 2015). The geologic range for *Perilimnadiidae* was fixed to late Permian to early Paleogene; we decided to use the upper boundary with an age of 252 Ma.

For each dataset, three independent Bayesian MCMC runs were carried out for 30–50 million of generations (to obtain effective sample size values of at least 200 for each parameter), to retain a sample of 10,000 trees. Convergence of the runs was confirmed using Tracer v1.6 software (<http://tree.bio.ed.ac.uk/software/tracer/>). The results of the three independent runs were then combined using LogCombiner v1.8.1 (with a burn-in of 25%), and MCC trees were generated using TreeAnnotator v2.1.2 and visualized using Fig-Tree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

## Results

### Spinicaudatan phylogeny

Phylogenetic reconstructions produced the same topologies for 40 representative spinicaudatan specimens using either maximum likelihood or Bayesian inference. Topologies confirm the monophyly of the Limnadiidae (Fig. 1). This family is divided into three groups: *Imnadia yeyetta*, *Limnadia lenticularis*, and all other limnadiids. Within the larger group, two clades are clearly and robustly distinguished. The first one is composed of Australian limnadiids, divided into a *Paralimnadia* clade and a *Limnadopsis* + *Australimnadia* clade. The second is composed of African and Neotropical limnadiids + *Eulimnadia* species. The new genus *BO sp. 1*, *Metalimnadia* (both strict Neotropical endemism) and all *Eulimnadia* species form a clade suggesting that *Eulimnadia* has probably



**Fig. 1** Phylogenetic tree of large branchiopod computed from the partial 28S rDNA (40 sequences) by Bayesian analysis (BI) and Maximum Likelihood (ML). To simplify, only the BI tree is shown; the ML tree has the same topology. The numbers are

an American origin. Within the *Eulimnadia*, two different groups seem to emerge with low resolution. Percentage of nucleotide identity of *BO sp. 1* with other genera are always under 96%, for example; 92–95% with *Metalimnadia* species, 93–96% with *Eulimnadia* species, 92–93% with *Gondwanalimnadia*, 95% with *Calalimnadia mahei*, 91–93% with *Limnadia lenticularis*, and 89–91% with *Imnadia yeyetta*.

Phylogenetic analysis of *Eulimnadia* genus

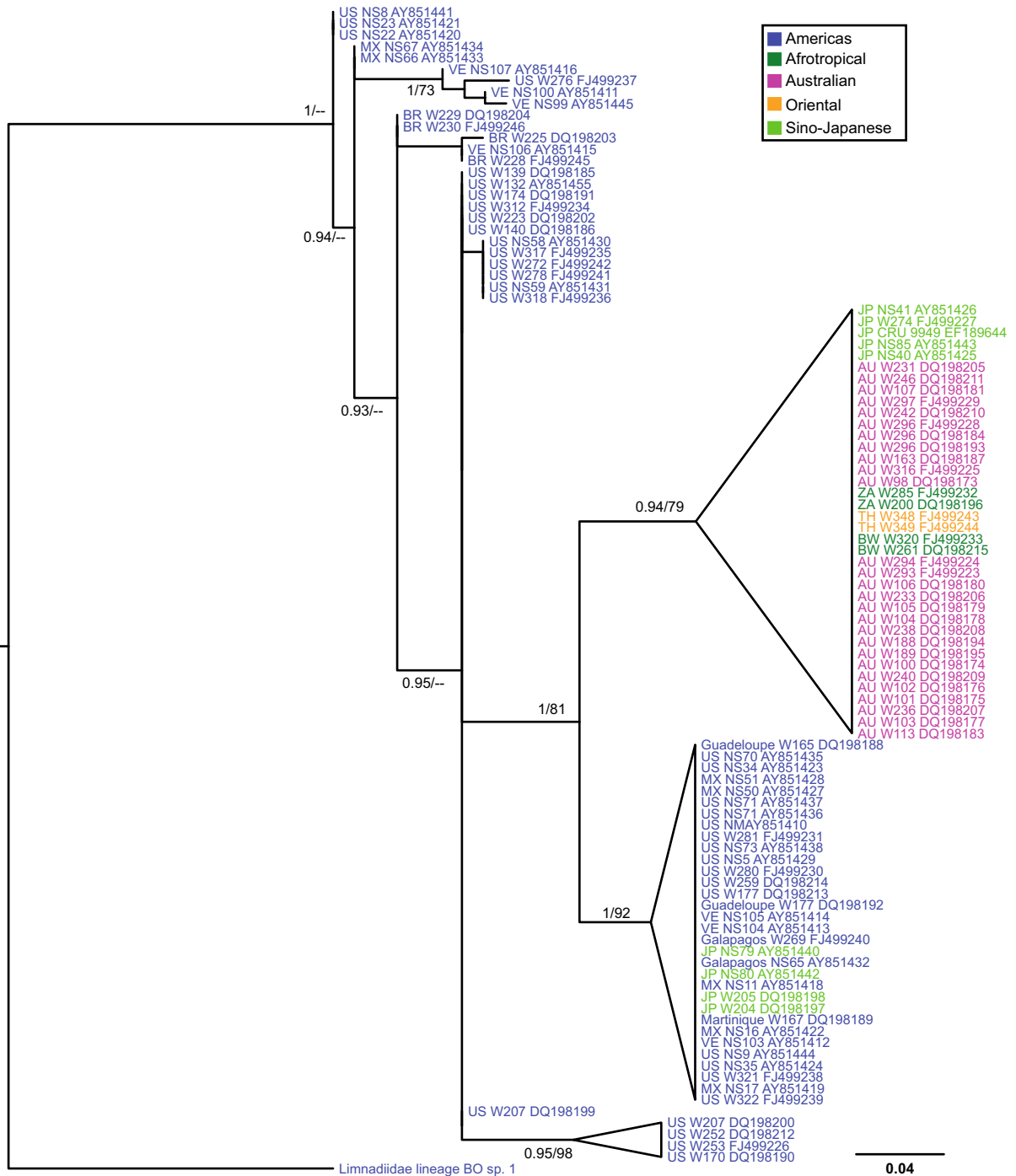
We produced the same topologies for 101 sequences of *Eulimnadia* (plus one outgroup) using either maximum likelihood or Bayesian inference (Fig. 2). We observed a clear pattern with all specimens from the Americas with basal positions. Results show an important cluster regrouping of all specimens from

posterior probabilities (BI) and bootstrap proportions (ML) reflecting clade support (values below 50 are indicated by *dashes*). Images of the new genus *BO sp. 1*; one male and one female (*bar scale* = 1 mm)

others geographic regions as Afrotropical, Oriental, Sino-Japanese, and Australian, except four specimens in Japan (identified as *E. cylindrova*).

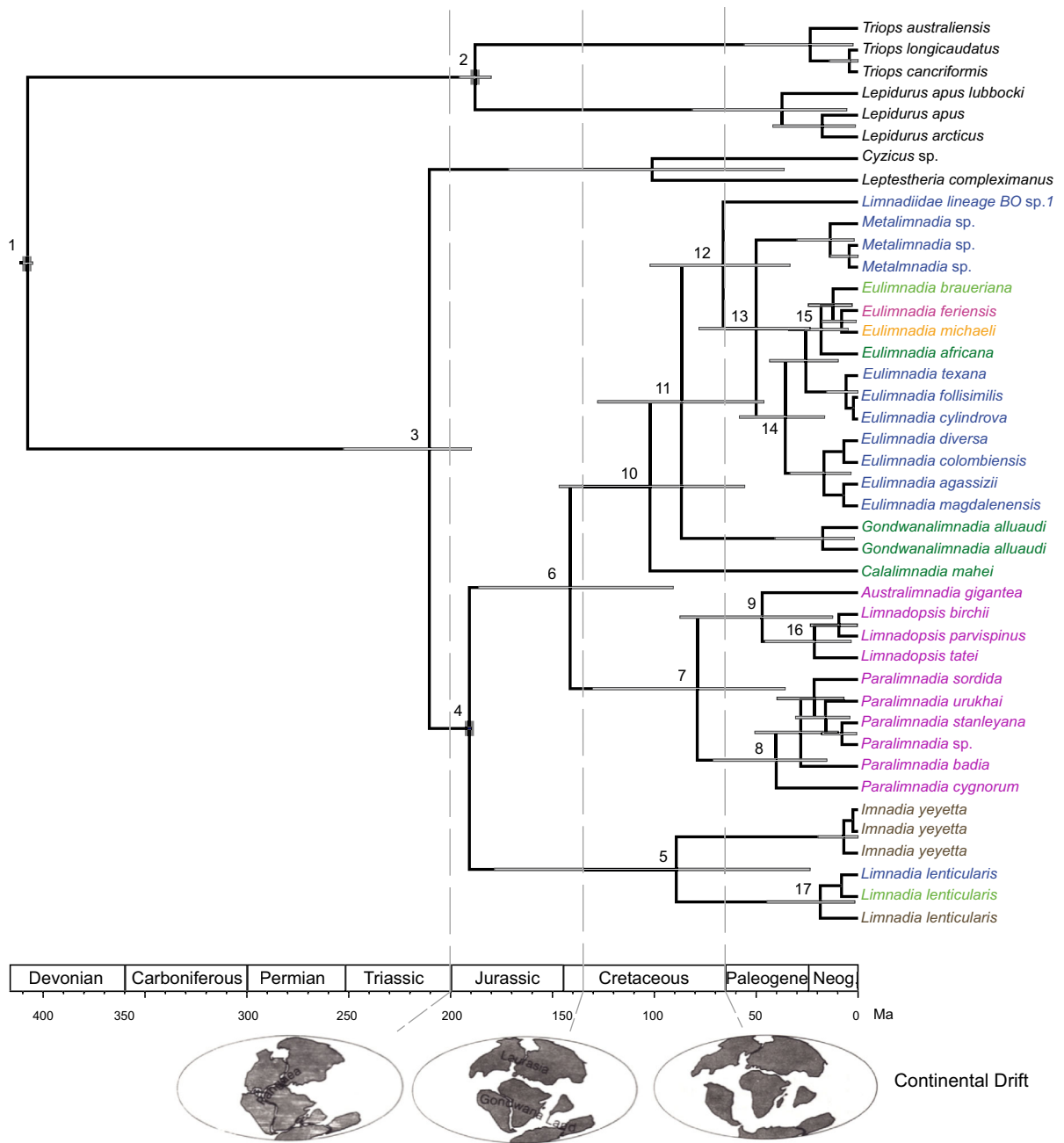
Divergence times

MCC trees with fossil calibration were constructed with two datasets. We illustrate only the results from the dataset containing 42 taxa (36 representatives species of Spinicaudata and six notostracan outgroups) with three calibrations (Fig. 3) but divergence dates of 17 nodes from both datasets are summarized in Table 2. In Fig. 3, we linked the MCC tree to geological stages and maps illustrating the continental drift. All analyses suggested an age between 192.9 and 295.7 Ma for the modern spinicaudatans. Pangaea was formed between the Devonian and the late Triassic, and divided into three main episodes (Scotese, 2001).



**Fig. 2** Phylogenetic tree of *Eulimnadia* specimens from the partial 28S rDNA (101 sequences) by Bayesian analysis (BI) and Maximum Likelihood (ML). To simplify, only the ML tree is shown; the BI tree has the same topology. The numbers are posterior probabilities (BI) and bootstrap proportions (ML)

reflecting clade support (values below 50 are indicated by dashes). Color codes show the geographic distribution of each sequence. Each specimen was named with the locality (international country code) plus isolate name and accession number. *Limnadiidae lineage BO sp. 1* was used as the outgroup



**Fig. 3** Time calibrated of 36 spini-caudatan species and 6 notostracan outgroups obtained in BEAST2 with 28S rDNA partial gene. Numbers at nodes identify divergence dates obtained and shown in Table 2. Gray square at nodes correspond to the calibrations given in M and M. Node bars correspond to the 95% highest posterior density (HPD) interval

of each node. Maps show the continental drift at different period of history. Geographical origin of Limnadiidae were represented as Afrotropical: green Australian: purple Oriental: orange Americas: blue Palearctic: brown and Sino-Japanese: light green

The break up began in the middle Jurassic (about 180 Ma), corresponding to an initial radiation for limnadiids with a split between laurasian limnadiids

(*Imnadia yeyetta* + *Limnadia lenticularis*) and gondwanian limnadiids (all others limnadiids, node 4). A second phase of the break up start in the early

**Table 2** Divergence date estimates (given in millions of years) for nodes in Fig. 3 and two other MCC trees with only one calibration

Nodes	3 calibrations	<i>Afrolimnadia</i> fossil calibration	<i>Perilimnadiidae</i> fossil calibration
1 Notostraca + Spinicaudata	407.6	ND	ND
2 Notostraca	188	ND	ND
3 Spinicaudata	210.5	192.9	295.7
4 Limnadiidae	190.8	191.4	252.4
5 Laurasian Limnadiids	89.2	77.4	44.1
6 Gondwanian Limnadiids	141.3	148.3	197
7 Australian Limnadiids	78.9	122.8	52.2
8 <i>Paralimnadia</i>	40.2	90.1	36.1
9 <i>Limnadopsis</i> + <i>Australimnadia</i>	47.1	75	43.2
10 West gondwana Limnadiids	102.2	106.5	142.6
11 Americas Limnadiids + <i>Gondwanalimnadia</i>	86.7	67.5	133
12 Limnadiids	66.2	50.9	84.2
13 <i>Eulimnadia</i> + <i>Metalimnadia</i>	50	39.2	51.2
14 <i>Eulimnadia</i>	35.7	32.4	47.7
15 Dispersive <i>Eulimnadia</i>	18.2	25.2	45.2
16 <i>Limnadopsis</i>	21.5	22.6	25.3
17 <i>Limnadia lenticularis</i>	18.6	20.4	36

For one analysis, a node age prior of 192 Ma for node 4 was calibrated with the fossil and the second one was calibrated with an age of 252 Ma

ND Not determined

Cretaceous (140 Ma) with the separation of the Antarctic/Australia and the Africa/South America blocks resulting in the partition of Australian limnadiids from the West Gondwanian lineages (node 6). The African lineage with *Gondwanalimnadia* and the Neotropical limnadiids + *Eulimnadia* divide during the continental separation of Africa and South America (node 11). In South America, as in Australia, during the Cretaceous to the Neogene the limnadiids diverge in several lineages (nodes 8, 9, 12, 13, 14). The expansion of *Eulimnadia* seems to be an exception and is not compatible with continental drift events. Emergence dates of *Eulimnadia* vary from 32.4, 35.7, to 47.7 Ma, clearly within the Paleogene. This genus presents an important diversity with three clades (node 14). Two of them are composed of *Eulimnadia* species coming from the Americas but the third seems to have colonized different continents. Estimated dates for this dispersive clade (i.e., *Eulimnadia* species from other geographical regions than the Americas) are 18.2, 25.2, or 45.2 Ma suggesting an American origin for them.

Dispersal capacity of limnadiids: the particular case of *Eulimnadia*

Six species of *Eulimnadia* were discovered on eight different oceanic islands far from continental landmasses (minimum of 80 km and maximum of 3,800 km) (Table 3). We calculated the minimal distance between islands (where specimens were detected); we found a minimum gap of 20 km for *E. texana* and a maximum of 1,000 km for *E. garretti*. These islands were never connected with continental mass in the past involving that *Eulimnadia* colonized islands through oceans.

Life cycle of limnadiids: the short life cycle of *Eulimnadia*

The life cycle and the time to reach maturity (Table 4) are usually short for *Eulimnadia* species compared to other limnadiids, particularly in tropical conditions (>27°C) where *Eulimnadia* species reach maturity 4–6 days after immersion. In Weeks et al. (1997) the



**Table 3** Island colonization by *Eulimnadia* species

<i>Eulimnadia</i> species	Island	Min. distance of continental mass	Presence of other Spinicaudata	References
<i>E. aff. geavi</i>	Barbados	220 km (Tobago Island)	No	MacKay & Williams (2011)
<i>E. aff. mauritiana</i>	Aldabra (Seychelles)	410 km (Madagascar)	No	Stoddart & Wright (1967), McKenzie (1971)
<i>E. cylindrova</i>	Martinique (French Caribbean island)	340 km (Tobago Island)	No	Weeks et al. (2006), Rabet et al. (2014)
<i>E. cylindrova</i>	Guadeloupe (French Caribbean island)	520 km (Tobago Island)	No	Weeks et al. (2006)
<i>E. cylindrova</i>	Santa Cruz (Galapagos)	1,030 km (South America)	No	Brendonck et al. (1990)
<i>E. garretti</i>	Huahine (French Polynesia)	3,800 km (New Zealand)	No	Richters (1882)
<i>E. mauritiana</i>	Mauritius	880 km (Madagascar)	Yes	Guérin (1837)
<i>E. texana</i>	St Johns (Virgin Island)	80 km (Puerto Rico)	No	Smith & Little (2003)

For each species, Island name and reference were indicated. Presence of other Spinicaudata was investigated and the minimal distance of the Island with a continental mass was calculated

time of 7 days is exceptional and the average age to become adult in this experiment is only 5,1 days. In similar conditions all the other genera reach maturity slower (6–10 days) and the life cycle is longer.

## Discussion

### Phylogenetic and divergence date estimate for Spinicaudata

This study is the first to combine phylogenetic reconstruction and divergence time estimates within Spinicaudata using fossil calibrations. The phylogenies show well-supported topologies with the monophyly of the *Limnadiidae*, which is consistent with previous studies (Hoeh et al., 2006; Schwentner et al., 2009). Inside this family, results show a group of Gondwanian species, the sister position of *Metalimnadia* with *Eulimnadia* and the association of *Gondwanalimnadia* and *Calalimnadia* to west gondwanian species (Weeks et al., 2006, 2009). Nevertheless, the topology changes in the west gondwanian group: *Calalimnadia* is the most basal group, and not *Gondwanalimnadia*, as was previously thought (Weeks et al., 2009). The limnadiid Bolivian sp. 1 appears to constitute a new lineage; the percentage of identity with other populations and phylogenetic isolation suggest a new genus. Indeed, species of limnadiids have only 2–3% of difference for the 28S ribosomal DNA whereas two genera have between 5

or 6% of difference. The new limnadiid Bolivian specimen will be described as a new genus and species in a future paper. The results also demonstrate an Australian clade (Schwentner et al., 2009) composed of all *Limnadopsis* and *Paralimnadia*. The monophyly of *Eulimnadia* is confirmed here but the topology inside the genus is not clear (Hoeh et al., 2006; Weeks et al., 2009). A recent molecular study of 19 *Eulimnadia* species also confirmed the monophyly of this genus and shows a low species level phylogenetic resolution (Reed et al., 2015). Our results and previous studies underscore the great need for a revision of *Eulimnadia*.

Some studies tried to estimate the age of branchiopod lineages, and each time only one or two sequences were used to represent Spinicaudata, introducing a massive methodological bias. Thus, Mathers et al. (2013) suggested a common ancestor for *Eulimnadia* sp. and *L. lenticularis* around 70 millions years ago which is inconsistent with our results. Another study gave an age estimation for Spinicaudata emergence of 266.3 Ma (183.1–307), which is consistent with our results (Korn et al., 2013). We were able to estimate the age of *Limnadopsis* (21–25 Ma); it is a little younger than what was reported previously (Schwentner et al., 2012). In the absence of *Limnadopsis* fossils, Schwentner et al. (2012) used an estimation of substitution rate for a mitochondrial gene (COI) suggested by decapod crustacean pairwise divergence rates, giving divergences dates with large confidence intervals. It is difficult to compare these

**Table 4** Life cycle and start of reproduction of different Limnadiids

Species name	Reproduction after immersion in days	Life cycle in days	Temperature	References
<i>Calalimnadia mahei</i>	7–10	≈ 40	28°C	This study
<i>Eulimnadia agassizii</i>	10*	18–19	Max 32°C in hot day	Berry (1926)
<i>Eulimnadia agassizii</i>	5–6	ND	32°C at 11 am	Zinn & Dexter (1962)
<i>Eulimnadia antlei</i>	4	ND	23–38°C	Belk (1972)
<i>Eulimnadia antlei</i>	11	ND	9–26°C	Belk (1972)
<i>Eulimnadia colombiensis</i>	4–5	15–20	28°C	This study
<i>Eulimnadia cylindrova</i>	5–6	20	28°C	This study
<i>Eulimnadia dahli</i>	5–7	ND	27°C	Benvenuto & Weeks (2011)
<i>Eulimnadia magdalenensis</i>	4–6	15–20	28°C	This study
<i>Eulimnadia texana</i>	4	ND	ND	Vidrine et al. (1987)
<i>Eulimnadia texana</i>	4–7*	22 (after 17 end of reproduction)	29°C	Weeks et al. (1997)
<i>Eulimnadia texana</i>	5–7	ND	27°C	Benvenuto & Weeks (2011)
<i>Imnadia yeyetta</i>	10	>30	15–25°C	Nourisson & Aguesse (1961)
<i>Imnadia yeyetta</i>	9	>30	20°C	This study
<i>Limnadia lenticularis</i>	15	>60	20°C	This study
<i>Limnadopsis tatei</i>	15	ND	27°C	Benvenuto & Weeks (2011)
<i>Metalimnadia</i> sp. lineage BR-PA	6–9	≈ 60	28°C	This study
<i>Paralimnadia badia</i>	10–12	ND	27°C	Benvenuto & Weeks (2011)
<i>Paralimnadia stanleyana</i>	8–9*	≈ 120	20°C (for reproduction)	Bishop (1968)

\* After hatching

studies because they did not explore the phylogeny of branchiopods at the same scale, with the same diversity or the same methods.

#### The particular case of *Eulimnadia*

Within the Limnadiidae, node ages and diversification patterns seem to perfectly fit with the Pangaea break up for most genera. This could be explained by a reduced capacity for transoceanic dispersal and a stepwise colonization via continental masses. Under this scenario, limnadiid populations, which were isolated after the continental drift, established new lineages progressively. If a limited dispersive capacity can be proposed for a majority of genera, this idea is in contradiction with the presence of *Eulimnadia* in

oceanic islands and their distribution. *Eulimnadia* has the largest distribution in this family, and it appears to be an exception with a high dispersive capacity. The colonization of oceanic islands (never in direct contact with continental mass) could only be explained by the transport of eggs. Until now, it was assumed that *Eulimnadia* was present 180 Ma and that it was widely distributed before the disconnection of the Pangaea (Weeks et al., 2006). But this assumption was not tested with calibrated phylogeny or evolutionary rates. Our work however shows an American origin of the genus *Eulimnadia* and a divergence date of 30 My.

Another important outcome of the study is the age of the *Eulimnadia* old world clade (i.e., *Eulimnadia* from Australia, Japan, Africa) around 20 Ma, which colonized the rest of the world. Indeed, some

populations, identified as *E. cylindrova* appeared in Japan suggesting that a second way of colonization of *Eulimnadia* could exist. In this case, the migration to Asia across the Pacific Ocean perhaps via the Galapagos Islands where the same species is also present probably occurred. However, we cannot exclude recent anthropogenic dispersal.

*Eulimnadia* are in part characterized by the absence of females and the presence of hermaphrodites and sometimes males, which is characteristic of androdioecy (Sassaman & Weeks, 1993; Weeks et al., 2006). Hermaphroditism seems to be a serious advantage for dispersal because only one egg we could produce one adult that will produce viable resting eggs, and a new population. Whereas gonochoric species must disperse many eggs to the same temporary pond in order to have a small probability to reproduce. Indeed, at least two eggs need to become opposite sex adults in the same time frame to support reproduction. Androdioecy presents the additional advantage that one egg can give a hermaphrodite heterozygote, which could produce genetic diversity within population by sexual recombination. These mixed descendants (hermaphrodite and male) will create diversity whereas exclusive hermaphrodites can form only clones. The resting eggs enhance the dispersive capacity of large branchiopods and two ways of dissemination are possible. First, by animals predated adults carrying the eggs (Mathias, 1936; Rogers, 2014) or by passive transport of laid eggs, for example when a pool dries out the wind spreads eggs (Graham & Wirth, 2008; Vanschoenwinkel et al., 2008). Due to the distance and the lack of biotope relay in the ocean, the long transoceanic colonization should be relatively rare.

Following our investigations on the limnadiid life cycle, tropical *Eulimnadia* have a short life cycle and reach maturity quickly. This ability is coupled to a high tolerance temperature, for example, Roessler (1995) indicated that tropical *Eulimnadia* can survive at 42°C. All these characteristics suggest that *Eulimnadia* is a pioneer selected to live in ephemeral and unstable ponds (<1 month). When *Eulimnadia* colonized other continents they compete with local limnadiids, which are characterized by a longer life cycle but they may establish in a specific ecological niche in temporary water. The monopolization hypothesis could explain this capacity of colonization and a rapid local differentiation (De Meester et al., 2002; Rogers, 2015).

Our investigation seems to confirm that most of limnadiids have a limited capacity of dispersal and most diversification in this family follows continental drift. One exception seems to be *Eulimnadia*, which seems to originate from the Americas and has a high dispersal capacity allowing it to colonize worldwide. Future investigation should focus on *Eulimnadia* with more sensitive phylogenetic markers in order to understand the initial radiation and the method of colonization.

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