

# The Evolution of Hermaphroditism from Dioecy in Crustaceans: Selfing Hermaphroditism Described in a Fourth Spinicaudatan Genus

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**Abstract** The evolution of reproductive systems has intrigued evolutionary biologists for well over a century. Recent empirical and theoretical work has elucidated the evolution of dioecy (separate males and females) from hermaphroditism in many plant species. The reverse transition, evolving hermaphroditism from dioecy, has occurred many times in animals, and yet is poorly studied relative to its reverse analog in plants. Crustaceans in the sub-order Spinicaudata have evolved hermaphroditism from dioecy three separate times, in some cases forming all-hermaphroditic species and in others forming androdioecious (males + hermaphrodites) species. Herein we report evidence of hermaphroditism in a fourth spinicaudatan genus: the newly described *Calalimnadia*. We present sex ratio and anatomical evidence that *Calalimnadia mahei* comprises selfing hermaphrodites, with no males being found in over 10,000 offspring reared. We combine these reproductive results with those of other Spinicaudata to estimate the evolution of hermaphroditism in this crustacean sub-order. We use these genetic data combined with anatomical evidence to suggest that *C. mahei* represents a fourth, independent derivation of hermaphroditism from dioecy in these reproductively labile crustaceans.

**Keywords** Sexual systems · Mating system · *Calalimnadia mahei* · Self-fertilization

## Introduction

The evolution of reproductive systems has intrigued evolutionary biologists since the earliest studies of evolution by natural selection (Darwin 1876). The vast array of reproductive modes (Bell 1982) has been exceptionally difficult to explain (Kondrashov 1993; de Visser and Elena 2007; Hadany and Comeron 2008; Lively 2010; Hartfield and Keightley 2012) with the majority of these explanations being abandoned for lack of support (Otto 2009). Thus, to this day, we continue to remain largely ignorant of the selective forces that maintain the broad range of reproductive modes found in the plant and animal kingdoms.

Nonetheless, we have made significant inroads into understanding some of the plethora of reproductive strategies and their evolution. One such area is the evolutionary transition between dioecy (gonochorism) and hermaphroditism (Barrett 2002; Charlesworth 2006). Evolutionary botanists have explored the evolution of dioecy from hermaphroditism in some detail (reviewed in Charlesworth 2006). The hermaphroditism to dioecy evolutionary transition has occurred dozens of times in flowering plants (Bawa 1980; Ashman 2002; Barrett 2010). Such a transition is thought to be driven by the avoidance of inbreeding depression in hermaphroditic species that commonly self-fertilize; mutants that lose the ability to produce one or the other gamete type are at an advantage because they do not waste energy on selfed offspring that experience high inbreeding depression (Charlesworth and Charlesworth 1978). Theory suggests that dioecy does not evolve from hermaphroditism directly but rather that a single-sexed

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mutant invades a hermaphroditic population and then spreads to temporarily coexist with hermaphrodites, forming either gynodioecy (females + hermaphrodites) or androdioecy (males + hermaphrodites). These predictions have been broadly tested and have been generally found to be correct (Charlesworth 2006).

The alternate evolutionary transition, from dioecy to hermaphroditism, has received much less attention, either theoretical or empirical (Weeks 2012). The commonly assumed selective pressure causing such a transition is termed “reproductive assurance” wherein self-compatible hermaphrodites are at an advantage over dioecious individuals in environments with low population density such that finding a mate might be difficult to impossible (Baker 1955; Ghiselin 1969). Outside of this, very little additional work has been done on the evolution of hermaphroditism from dioecy, almost all being theoretical (Ghiselin 1974; Charnov 1982) and very little empirical (reviewed in Weeks 2012). There are approximately 65,000 animal species that are known to employ hermaphroditism (Jarne and Auld 2006), with possibly the majority of these species evolving from dioecious ancestors (Ghiselin 1969; Eppley and Jesson 2008; but see Iyer and Roughgarden 2008 for an alternate view). Given the large number of hermaphroditic animal species, more attention to the dioecy to hermaphroditism evolutionary transition in animals is certainly warranted (Weeks 2012).

One taxon that has shown repeated evolution of hermaphroditism from dioecy is the crustacean suborder Spinicaudata. Weeks et al. (2009) describe two independent origins of hermaphroditism from dioecy: one in the genus *Limnadia*, and one in the genus *Eulimnadia*. In the former, the hermaphrodites outcompeted both males and females to form the all-hermaphroditic species *Limnadia lenticularis*. For the latter, hermaphrodites outcompeted females, but not males, to form androdioecious (males + hermaphrodites) species. Recently, a third, independent derivation of hermaphroditism from dioecy was documented in a separate spinicaudatan family (Cyzicidae): *Cyzicus gynecia* (Brantner et al. 2013a). Clearly, Spinicaudata are reproductively labile and prone to transitions from dioecy to hermaphroditism, and thus this crustacean taxon is a logical group in which to study this important evolutionary transition.

Herein we describe evidence that a species in a fourth genus, the newly described *Calalimnadia mahei* (Rogers et al. 2012), also is all-hermaphroditic. We provide sex ratio information from isolated *C. mahei* that show no male production was documented in over 10,000 offspring reared, and also show conclusive evidence that *C. mahei* produces both sperm and eggs in an ovotestis. We then describe the likely evolution of hermaphroditism within the Spinicaudata and discuss the ramifications of this transition.

## Methods and Materials

### Rearing from Egg Banks

The resting eggs of clam shrimps were collected in soil samples from a dry temporary pool. Clam shrimp resting eggs (“cysts”) are produced by females/hermaphrodites directly after sexual maturity and then throughout their lifespans on a daily or bi-daily basis (Weeks et al. 1997). Branchiopod cysts can remain viable in soil for decades or longer (Dumont and Negrea 2002), and any clutch of eggs can have some hatching over repeated hydrations (Simovich and Hathaway 1997). Thus, the eggs in the soil collections likely encompassed a broad range of ages. Resting eggs of *C. mahei* were collected from Mauritius Island, Cap Malheureux from a pool called “La Mort” (Rogers et al. 2012) and transported to France for initial rearing. Egg banks were developed in France from these initial rearings of 10 specimens from La Mort and were then sent to Akron to establish a lineage for study.

The egg banks produced above were hydrated by adding deionized (DI) water, and fed daily with 1 mL of a mixture composed of DI water (500 mL) and baker’s yeast/Tetramin™ flake food (Tetra Werke, Melle, Germany; 2.5 grams each). All hydrated egg bank cups were kept in continuous light and maintained at temperatures ranging between 25 and 28 °C. Approximately 2 days following hydration, resulting nauplii were transferred to ‘rearing tanks’ prepared as previously described (Brantner et al. 2013a), which were maintained under ‘standard growth conditions’ (Sassaman and Weeks 1993; Weeks et al. 1997). Approximately 5 days post-hydration of the egg banks, 52 immature clam shrimp were individually placed into separate 500 mL cups. Sexual maturity was observed (i.e., appearance of eggs in the brood chamber of hermaphrodites) in clam shrimp in both the aquaria and the isolation cups approximately 6–10 days post the initial appearance of nauplii in the egg bank cup hydrations. Sex ratios were documented for both isolated and aquarium-reared shrimp and all shrimp were frozen after sexual maturity, except those taken for microscopy (see below).

These 52 cups were allowed to dry for 30 days or more. Deionized water was then added to 27 cups, and 23 hatched. When nauplii were seen in the rearing cups, the nauplii were transferred to a 10 L plastic “rearing tanks,” as noted above. The nauplii were reared as outlined above and then sexed at sexual maturity.

### Microscopy

Clam shrimp were prepared for microscopy using detailed methods described elsewhere (Brantner et al. 2013a, b). Briefly, clam shrimp specimens were removed from rearing

tanks upon sexual maturity. The samples were pre-fixed in 2 % glutaraldehyde buffered with 0.1 M sodium cacodylate (pH = 7.2) for 2 h, carapaces were removed, and the soft bodies were fixed further for an additional 1 h in 2 % glutaraldehyde/0.1 M sodium cacodylate (pH = 7.2) solution. Samples were washed with 0.1 M sodium cacodylate (pH = 7.2) for 1 h. Specimens were post-fixed in 2 % osmium tetroxide (OsO<sub>4</sub>) buffered with 0.1 M sodium cacodylate (pH = 7.2) for 1.5 h, and then washed with DI water for 0.5 h. Samples were *en bloc* stained with 2 % uranyl acetate for 0.5 h, and then washed for 0.5 h with DI water. Clam shrimp were dehydrated overnight using an acetone desiccator (Ott and Brown 1974), and were further subjected to plastics processing (Embed-812; Electron Microscopy Science, Hatfield, PA, USA). Embedded clam shrimp were sectioned using a diamond knife mounted on a Reichert OMU-3 ultramicrotome. Thick sections of approximately 1.5 μm were heat fixed onto glass slides, stained with a 1 % toluidine/sodium borate solution, and photographed using an Olympus DP71 digital camera system mounted onto an Olympus BX60 digital light microscope (Olympus America Inc., Center Valley, PA, USA). Thin sections (approximately 0.2 μm) were placed on copper mesh grids, pre-stained in 1 % methanolic uranyl acetate, and post-stained in Reynolds' lead citrate (Reynolds 1963). Thin sections were viewed and imaged at the Molecular and Cellular Imaging Center (MCIC) at the Ohio Agricultural Research and Development Center (OARDC) with a Hitachi H-7500 transmission electron microscope (Hitachi High-Technologies, Hitachinaka, Japan).

#### Phylogenetic Reconstruction

28S ribosomal data was retrieved from GenBank for 42 spinicaudatan species and for the Notostracan *Triops longicaudatus* for use as an outgroup. Data were aligned using CLUSTAL W 1.82 (Thompson et al. 1994) via the online server CIPRES Science Gateway (Miller et al. 2010). Alignments were assessed and trimmed by eye in Mesquite (Maddison and Maddison 2007) to congruent sequences of 800 bp length.

For Bayesian Inference (BI), all runs were performed using MrBayes v.3.2.1 (Huelsenbeck and Ronquist 2001) at 1,000,000 generations (mcmc) for each run with 100,000 generations discarded as burn-in. We used standard settings and four chains for both parallel runs in MrBayes. Each chain was sampled every 100 generations. Each run was examined to ensure likelihood stability during burn-in and consistency between runs.

Maximum Likelihood (ML) analyses were performed using the R-package Phangorn (Schliep 2011). The best fit model of sequence substitution was deduced using distance-based topologies (NJ, UPGMA) with the hierarchical

likelihood ratio tests performed via the modelTest function in Phangorn. Bootstrapping using 1,000 replicates was implemented in all ML analyses.

#### Ancestral State Reconstruction

The presence/absence of ancestral hermaphroditism was inferred using BayesTraits v. 1.0 (Pagel and Meade 2007) using four different models of character evolution. Markov chains were run for 10 million generations, sampling every 1,000 generations with a burn-in of 10,000. We assumed uniform priors with default upper and lower bounds for all models. Sequential alterations to the model along the chain were limited using the *ratedev* command to produce acceptance rates of approximately 20–40 %.

## Results

#### Sex Ratios

No males were found among the 38 shrimp originally collected in the field from Mauritius Island. From the original egg banks collected in France from 10 of these 38 shrimp, 52 shrimp matured as all-“female.” (Note: sex is described as “female” because simple morphological assessment cannot distinguish females from hermaphrodites; see below.) Each of these “females” was isolated and 23 of these 52 had their eggs hatched and reared to maturity (Table 1). From these 23 “females,” a total of 10,038 offspring were reared to maturity and sexed. 100 % of these 10,038 offspring developed as “female.”

#### Histology and Cell Ultrastructure

Sexually mature *C. mahei* “females” were longitudinally and transversally sectioned. Posterior examination of the “female” tubular gonad (two lobes situated in the hemocoel running adjacent to and parallel with the digestive tract) displayed structural similarity to that of other spinicaudatan female gonads. A thick female wall composed of epithelial cells and female germinal cells surround a central lumen (Fig. 1). Female germ cells mature away from the lumen of the gonad towards the hemocoel. Following female germ cell maturation into developed oocytes (recognized by abundant vitelline droplets), the oocytes descend back into the central gonad lumen, where a dense, darkened material (eggshell secretory matter) secreted by glandular epithelial cells of the female wall encases the mature oocytes. Musculature surrounding the gonad facilitates oocyte motion through the lumen of the gonad tract (Fig. 1). No evidence of testicular tissue was identified in the posterior region of the gonad.

**Table 1** Offspring and sex ratios from 23 isolated *C. mahei* “females”

Isolate #	Females	Males
A1	265	0
A2	5	0
A3	471	0
A4	203	0
A5	567	0
A6	5	0
A7	1,228	0
A8	1,196	0
A9	696	0
A11	275	0
A12	360	0
A13	26	0
A14	348	0
A15	87	0
A16	1,550	0
A17	1,150	0
A18	462	0
A20	39	0
A21	535	0
A22	58	0
A23	374	0
A24	131	0
A25	7	0
Total	10,038	0

Examination of the anterior portion of the “female” gonad of *C. mahei*, however, revealed structural heterogeneity. The anterior-most tip of the bi-lobed tubular gonad (situated directly behind the head region) consisted of a well-defined male wall region (immediately adjacent to the female wall) from which non-flagellated, amoeboid male gametes were visualized moving into the luminal space of the gonad (Figs. 1b, c, 2a). The male wall was composed of thinly stretched epithelial tissue and inter-mixed male gametes (Fig. 2a, b). Upon moving into the luminal space, male gametes come into contact with egg-shell secretory matter (Fig. 1b). Early developing male gametes still attached to or near the male wall appeared cytoplasmically dense and contained loosely compacted nuclear material (Fig. 2a), whereas many mature male gametes in the gonad lumen possessed condensed chromatin blocks against the inner nuclear membrane (Fig. 2b). Intercellular bridges connecting male gametes were also noted in developing male gametes still attached to the male wall (Fig. 2a).

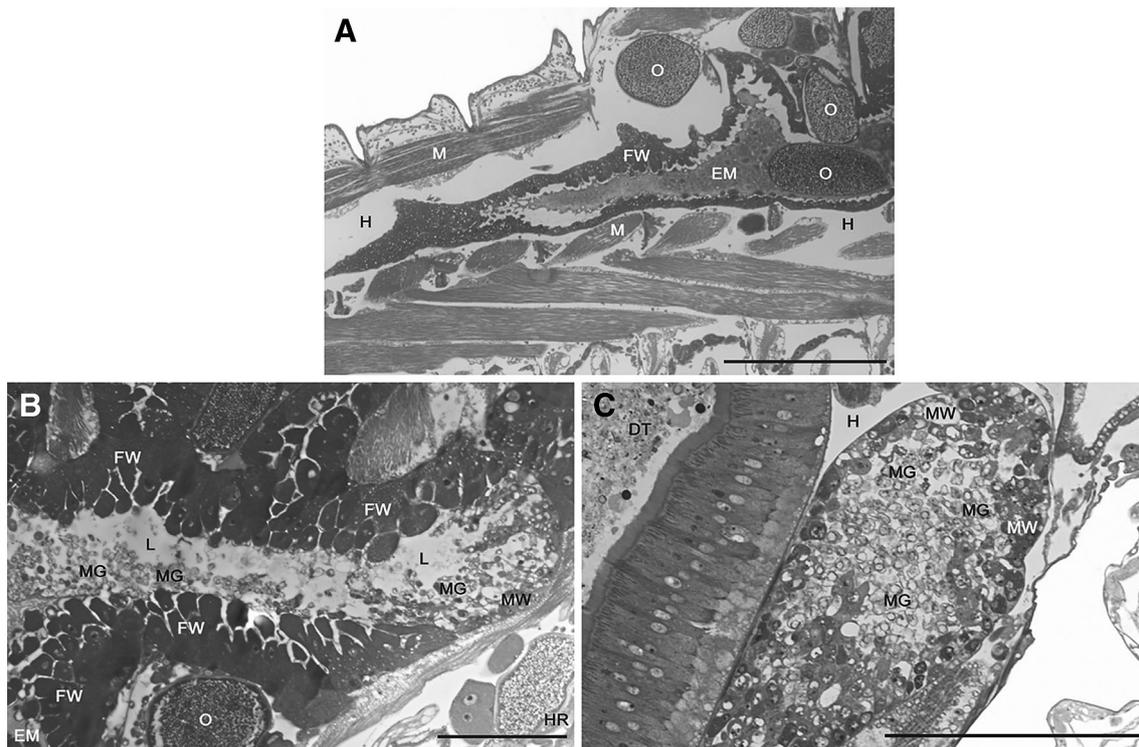
These combined sex ratio and anatomical results clearly show that *C. mahei* “females” are, in fact, hermaphrodites.

## Phylogeny

Bayesian inference of the 28S data for all 43 taxa (Table 2) produced 2001 trees, the 50 % majority rule consensus of which matches the topology produced by the maximum likelihood analysis (Fig. 3). This topology is congruent with other, more inclusive phylogenetic hypotheses suggested in other studies of the Spinicaudata (Richter et al. 2007; Schwentner et al. 2009) in that the monophyly of the Limnadiidae and Cyzicidae are apparent. *Paralimnadia* and *Limnadopsis* form an endemic Australian clade as previously recovered by Schwentner et al. (2009). It is likely that *Caenestheriella* and *Caenestheria* are synonymous with *Cyzicus*; subsequently the monophyly of *Cyzicus* is supported, but the relationship between *Cyzicus* + *Eocyzicus* + *Leptestheria* is uncertain. Bootstrap scores strongly support the positions of *Calalimnadia*, *Afrolimnadia* and *Metalimnadia* as basal to *Eulimnadia* (respectively), the monophyly of which is also strongly supported.

Assigning known occurrences of hermaphroditism to 40 of the spinicaudatan taxa for which 28S sequences had been collected [*Limnadia nipponica* (*lenticularis*) is omitted due to suggested synonymy with *L. lenticularis* (Straskraba 1964); *Afrolimnadia alluadi* is omitted as this species’ sexual system is currently unknown] allowed us to explore likely models of evolution for this ‘trait’ using the 2,001 trees produced by MrBayes in BayesTraits. For this comparison, “androdioecy” (i.e., mixtures of males and hermaphrodites) was considered “hermaphroditic” for purposes of the evolution of hermaphroditic forms. As this trait was coded in a binary fashion (0 = absence, 1 = presence of hermaphroditism), four possible models of trait evolution were available to test. Model A represents different, unrestricted rates of transition both to and from hermaphroditism; model B represents a fixed single rate of transition to and from hermaphroditism; model C represents unrestricted transitions to hermaphroditism but disallowing transitions back; and model D represents a system where only transitions from hermaphroditism are allowed. The results of testing each model, given the data produced by Bayesian inference, can be seen in Table 3. Although model B appears to fit the data better than either A or C, the small difference in Bayes factor between these models suggests that none of these three models are significantly more likely than the other; however the considerably lower likelihood of model D allows us to rule out the absence of transitions to hermaphroditism.

The likelihood of individual lineages arising from ancestral populations that were either dioecious (black) or contained self-fertilizing hermaphrodites (grey; Fig. 4) are displayed as individual pie-charts at each node. The ‘strength’ of a likelihood prediction for a certain node can



**Fig. 1** Transverse and longitudinal thick sections of *C. mahei*. **a** Posterior-most region of one lobe of the bi-lobed gonad. The central gonad lumen (*L*) is surrounded by a thick female wall (*FW*). Mature oocytes (*O*) become encased in eggshell secretory matter (*EM*) upon descent into the gonad lumen. **b** Anterior view of the gonad located

directly behind the head region (*HR*). Male gametes (*MG*) develop away from a thin male wall (*MW*) towards the gonad lumen. **c** Transverse section of the male wall region of the gonad with male gametes at various developmental stages. *DT* digestive tract, *H* hemocoel, *M* striated muscle. Scale bars 500  $\mu\text{m}$  (**a**), 200  $\mu\text{m}$  (**b**, **c**)

be assessed by the predominance of either state at that node. In this respect it can be predicted that, given the model, the ancestral spinicaudatan arose from dioecious ancestors and that self-fertilizing hermaphroditism arose independently in the lineages leading to *L. lenticularis*, *C. gynecia* and the *Calalimnadia* + *Metalimnadia* + *Eulimnadia* lineage. The occurrence of a transition back to dioecy from hermaphroditism is suggested for *Metalimnadia*.

## Discussion

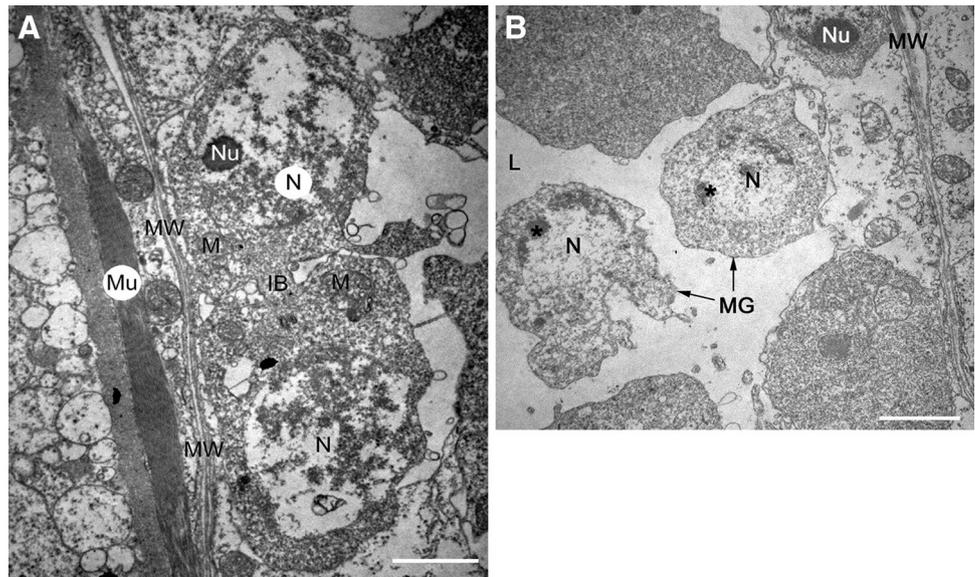
Many researchers have studied the transitions between breeding systems to better understand the evolution of reproductive systems more generally (Charlesworth 2006; Jarne and Auld 2006; Eppley and Jesson 2008; Iyer and Roughgarden 2008; Weeks 2012). An understudied transition is that of hermaphroditism evolving from dioecy (Weeks 2012), which appears to have evolved numerous times within the Animalia (Eppley and Jesson 2008). The Spinicaudata are a taxon which has made such a transition at least three separate times (Weeks et al. 2009; Brantner et al. 2013a), and thus are an interesting set of species to

explore further to both examine the extent of these transitions as well as the possible causes of these evolutionary changes.

The offspring rearings and anatomical evidence clearly show that selfing hermaphroditism occurs in yet another genus in the Spinicaudata: the newly described *Calalimnadia* (Rogers et al. 2012). No males were found in the field and no males were reared from eggs in the lab in Paris or in Akron. Among 23 isolated “females,” over 10,000 offspring were reared to sexual maturity; not a single male was discovered for any of these 10,000+ offspring (Table 1). This finding suggests that sex is not environmentally sensitive but is rather genetically fixed, as has been found for all of the clam shrimp species studied so far (Sassaman and Weeks 1993; Weeks et al. 2008, 2010). Weeks et al. (2008) suggest that sex ratios reliably in the 0–5 % male range are either all-hermaphroditic species or are isolated “monogenic” hermaphrodites from an otherwise androdioecious species. In either case, species with sex ratios in the 0–5 % range have been reliably typed as hermaphroditic, out of the >23 clam shrimp species reproductively examined to date (Weeks et al. 2008).

Additionally, when these likely hermaphrodites were sectioned, their gonads clearly contained oocytes and male

**Fig. 2** Transmission electron micrograph of the male wall (MW) region of *C. mahei*. **a** Two male gametes (MG) connected via an intercellular bridge (IB) still attached to the male wall. **b** Male gametes found in the lumen (L) of the gonad often possessed highly compacted chromatin blocks against the inner nuclear membrane (asterisk). M mitochondria, Mu striated muscle, N nucleus, Nu nucleolus. Scale bar 2  $\mu$ m



gametes occurring concomitantly in the reproductive tract, confirming their hermaphroditism. Both gamete types were similar to gametes described in other spinicaudatan species. The development of the oocytes, the eggshell secretory matter and the epithelial cells of the female wall directly resemble the female gonads described in other Spinicaudata (Tommasini and Scanabissi Sabelli 1992; Zucker et al. 1997; Scanabissi and Mondini 2002a, b; Weeks et al. 2005; Brantner et al. 2013a, b). The non-flagellated, ameboid male gametes are also similar to spermatozoa described from other Spinicaudata (Zucker et al. 1997; Scanabissi and Mondini 2002a; Weeks et al. 2005; Scanabissi et al. 2006; Brantner et al. 2013a, b). These similarities in gonad structure among species, combined with the sex ratio data above, verify that *C. mahei* is a self-compatible hermaphrodite, and is now the fourth genus of Spinicaudata to contain one or more species that reproduce in this way (the other three being *Limnadia*, *Cyzicus*, and *Eulimnadia*).

Although the general form of the gametes is the same as in other spinicaudatan species, the location of the testicular tissue is not consistent among all of the hermaphroditic species. *L. lenticularis* hermaphrodites have been shown to possess testicular tissue haphazardly scattered throughout the tubular gonad (Zaffagnini 1969; Scanabissi and Mondini 2002b). In all six of the *Eulimnadia* hermaphroditic species so far examined (*E. agasizii*, *E. azisi*, *E. gibba*, *E. guntur-ensis*, *E. michaeli*, and *E. texana*), the testicular tissue has been restricted to the posterior tip of the ovotestis (Zucker et al. 1997; Weeks et al. 2005; Brantner et al. 2013b). In the recently described hermaphroditic *C. gynecia*, the testicular tissue is located in the anterior tip of the gonad (Brantner et al. 2013a). Herein, the testicular tissue in *C. mahei* ovotestes was also located just behind the head region (Fig. 1b). The three separate locations for *Eulimnadia*,

*Limnadia* and *Cyzicus* are consistent with three separate derivations of hermaphroditism from dioecy (Brantner et al. 2013a). Having a second genus (*Calalimnadia*) with testicular tissue located behind the head could signify that both species are derived from a singular progenitor, or that there is simply not many locations that such tissue could locate, and thus could be convergent evolution of a similar location by separate, unique derivations. As we will argue below, the latter seems the most parsimonious scenario.

The phylogenetic analyses conducted in this study produced stable topologies that are highly congruent with other studies that use multiple genes (Fig. 3). The monophyly of established families are supported and the relationships among genera within the Limnadiidae, Cyzicidae and Leptestheriidae are resolved with confidence. The position of the group *Imnadia* + *Limnadia* as basal to the Limnadiidae is of interest as these lineages not only appear long-lived but represent both dioecy (in *Imnadia*) and hermaphroditism (in *Limnadia*). The occurrence of the monospecific genera *Calalimnadia*, *Afrolimnadia* and *Metalimnadia* as basal to the *Eulimnadia* lineage is of particular interest.

The derivation of an appropriate model for the evolution of hermaphroditism in this study assumed a binary state for either hermaphroditism or dioecy to express the presence/absence of sperm production in females. It is possible that this model may be over-simplified (and subsequently be the cause of neither model A, B or C being statistically preferred, Table 3) and that a technique that takes into account known biological dynamics of sexual system transition (e.g., allowing the consideration of androdioecy as a transitional state between dioecy and hermaphroditism) needs to be developed to provide a more precise explanation of the evolution of hermaphroditism in this group.

**Table 2** Taxa used in this analysis, their GenBank ascension numbers and the original study the sequence data came from

Genus	Species	28S GenBank accession no.	Publication
<i>Leptestheria</i>	<i>compleximanus</i>	AY851398	Hoeh et al. (2006)
	<i>dahalacensis</i>	EF189648	Richter et al. (2007)
	<i>kawachiensis</i>	EF189649	Richter et al. (2007)
<i>Caenestheria</i>	<i>lutraria</i>	EF189639	Richter et al. (2007)
<i>Caenestheriella</i>	<i>gifuensis</i>	EF189640	Richter et al. (2007)
<i>Cyzicus</i>	<i>setosa</i>	AF532879	Taylor et al. (1999)
	<i>californicus</i>	FJ830362	Schwentner et al. (2009)
	<i>gynecia</i>	AY851403	Hoeh et al. (2006)
<i>Eocyclus</i>	<i>tetracerus</i>	FJ830363	Schwentner et al. (2009)
	<i>argillaquus</i>	FJ830364	Schwentner et al. (2009)
	<i>digueti</i>	AY851407	Hoeh et al. (2006)
<i>Limnadia</i>	<i>parooensis</i>	FJ830365	Schwentner et al. (2009)
	<i>mongolianus</i>	JN205065	Sun et al. (2006)
	<i>orientalis</i>	JN205066	Sun et al. (2006)
	<i>serratura</i>	AY851417	Hoeh et al. (2006)
	<i>africana</i>	DQ198215	Weeks et al. (2006)
	<i>agassizii</i>	AY851431	Hoeh et al. (2006)
	<i>braueriana</i>	EF189644	Richter et al. (2007)
	<i>colombiensis</i>	AY851414	Hoeh et al. (2006)
	<i>brasiliensis</i>	DQ198203	Weeks et al. (2006)
	<i>cylindrova</i>	AY851432	Hoeh et al. (2006)
<i>Calalimnadia</i>	<i>dahli</i>	DQ198194	Weeks et al. (2006)
	<i>diversa</i>	AY851441	Hoeh et al. (2006)
	<i>feriensis</i>	DQ198211	Weeks et al. (2006)
	<i>packardiana</i>	AY851443	Hoeh et al. (2006)
	<i>texana</i>	AY851437	Hoeh et al. (2006)
	<i>follisimilis</i>	FJ499238	Weeks et al. (2009)
	<i>magdalenensis</i>	AY851445	Hoeh et al. (2006)
	<i>michaeli</i>	FJ499244	Weeks et al. (2009)
	<i>mahei</i>	AY851439	Hoeh et al. (2006)
	<i>yeyetta</i>	EF189646	Richter et al. (2007)
<i>Limnadia</i>	<i>lenticularis</i>	EF189651	Richter et al. (2007)
	<i>lenticularis (nipponica)</i>	FJ499286	Weeks et al. (2009)
<i>Limnadopsis</i>	<i>birchii</i>	FJ830369	Schwentner et al. (2009)

**Table 2** continued

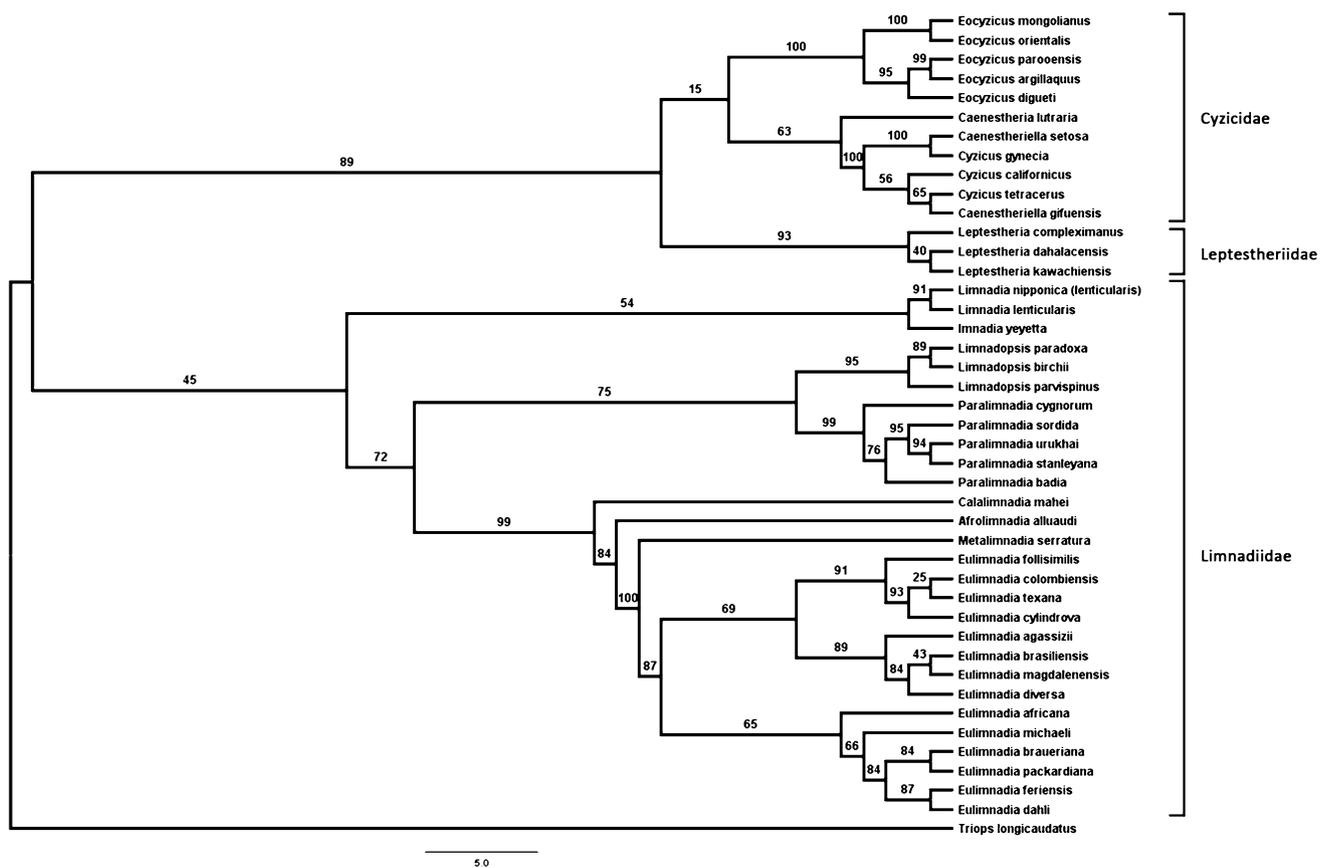
Genus	Species	28S GenBank accession no.	Publication
	<i>parvispinus</i>	FJ830370	Schwentner et al. (2009)
	<i>paradoxa</i>	FJ830372	Schwentner et al. (2009)
<i>Paralimnadia</i>	<i>urukhai</i>	FJ499268	Weeks et al. (2009)
	<i>stanleyana</i>	FJ499270	Weeks et al. (2009)
	<i>sordida</i>	FJ499278	Weeks et al. (2009)
	<i>cygnorum</i>	FJ499272	Weeks et al. (2009)
<i>Afrolimnadia</i>	<i>badia</i>	FJ499261	Weeks et al. (2009)
	<i>alluaudi</i>	FJ499251	Weeks et al. (2009)
<i>Triops</i>	<i>longicaudatus</i>	AM269436	Korn and Hundsdoerfer (2006)

### Evolution of Hermaphroditism in the Spinicaudata

Weeks et al. (2009) noted two separate derivations of hermaphroditism (in *L. lenticularis* and in the progenitor to the *Eulimnadia*) and one instance of asexual reproduction (*C. gynecia*) in the Spinicaudata. They also found that the progenitor to the Spinicaudata was likely dioecious, and thus that the above derivations were from dioecy to hermaphroditism/asexuality. Since that study, Brantner et al. (2013a) have definitively shown that *C. gynecia* is actually all-hermaphroditic rather than asexual. Mistaking all-hermaphroditic species for asexuals is a common problem in the Branchiopoda (Sassaman 1991, 1995; Sassaman and Weeks 1993), and thus finding that *C. gynecia* is, in fact, hermaphroditic rather than asexual is not surprising.

Herein, then, we have a fourth genus, *Calalimnadia*, which is now known to be all-hermaphroditic. The logical follow up question to this finding is whether *Calalimnadia* is a unique derivation of hermaphroditism or whether it is reflective of a deeper common ancestor to one or more of the other hermaphroditic taxa.

The current phylogenetic reconstruction established via BayesTraits (Table 3) of the 40 spinicaudatan taxa for which we have reproductive data (we have no reproductive data on *Afrolimnadia alluaudi*) suggests three separate derivations of hermaphroditism: one for *C. gynecia*, one for *L. lenticularis* and one for the ancestor to the *Calalimnadia* + *Metalimnadia* + *Eulimnadia* (Fig. 4). Using this model, it appears that the transition to hermaphroditism in *Calalimnadia* occurred in the progenitor to *Calalimnadia* + *Metalimnadia* + *Eulimnadia* (Fig. 4). As the calculated probabilities of the ancestral state at the nodes for



**Fig. 3** Maximum likelihood analysis of 28S DNA sequence data of 42 spinicaudatan species and one Notostracan outgroup. Bootstrap values are above the branches

**Table 3** Models of transition between dioecy & hermaphroditism investigated using BayesTraits

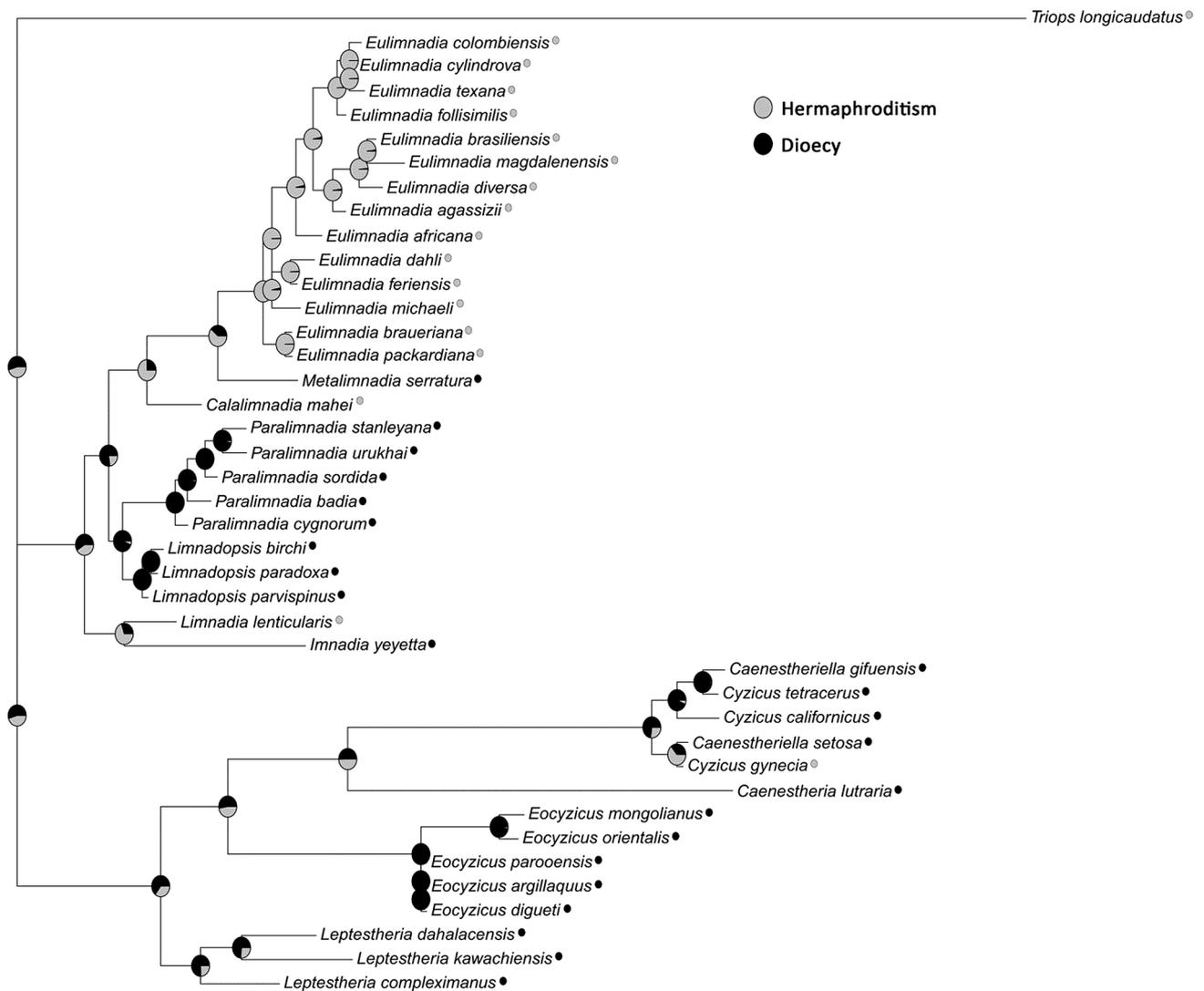
Model	q01	q10	−ln L	ln harmonic mean L	2 ln Bayes factor
A	19.62 (±0.24)	29.78 (±0.33)	−16.95 (±0.03)	−18.83 (±0.00)	2.58
B	16.93 (±0.21)	16.93 (±0.21)	−16.25 (±0.02)	−17.54 (±0.00)	0.00
C	7.90 (±0.06)	0	−17.17 (±0.02)	−18.64 (±0.00)	2.19
D	0	13.45 (±0.09)	−21.81 (±0.03)	−23.71 (±0.00)	12.34

q01 dioecy to hermaphroditism, q10 hermaphroditism to dioecy. −ln L, log likelihood of model. The 2 ln Bayes factor can be used to assess the viability of other models compared to the best performing model

*C. mahei* and *M. serratura* show only partial favor of hermaphroditism over dioecy, it remains uncertain whether a transition occurred back from hermaphroditism to dioecy in the *Metalimnadia* lineage or one transition to hermaphroditism occurred in *Calalimnadia* and another separate transition to hermaphroditism occurred later in *Eulimnadia* with dioecy remaining in *Metalimnadia* as an ancestral character (Fig. 4).

Weeks et al. (2009) note that the evolution of androdioecy from dioecy is more flexible than that of all-hermaphroditism from dioecy, because both the Z and W chromosomes are retained in androdioecy allowing either all hermaphroditism (WW) or dioecy (ZW + ZZ) to

evolve from an androdioecious ancestor. When a lineage evolves all hermaphroditism, the male-specific genes on the Z chromosome have been lost (through the fixation of the W chromosome in WW hermaphrodites), and thus re-establishment of fully male individuals should be exceptionally improbable (Weeks et al. 2009). Thus, it is conceivable that the ancestor to the *Calalimnadia* + *Metalimnadia* + *Eulimnadia* was androdioecious, and that *Calalimnadia* derived all-hermaphroditism while *Metalimnadia* derived dioecy from this androdioecious ancestor. In this scenario, *Eulimnadia* would have retained the ancestral breeding system of androdioecy.



**Fig. 4** 50 % majority-rule consensus tree of 2001 trees produced by the Bayesian analysis of 28S DNA sequence data. Pie charts at nodes illustrate the probability of the ancestral state being dioecy (black) or

hermaphroditism (light gray). Colored circles to the right of the species names denote breeding system for each species

We feel that another scenario is more likely: that the hermaphroditism in *Calalimnadia* is actually separately derived from that seen in *Eulimnadia* and thus represents a fourth derivation of hermaphroditism within the Spinicaudata. We base this assertion on the location of the testicular tissue in *Calalimnadia*, *Limnadia* and *Eulimnadia*. Within all six *Eulimnadia* species examined to date, all show the same location of the testicular tissue: at the posterior-most section of the ovotestes (Zucker et al. 1997; Weeks et al. 2005; Brantner et al. 2013b). On the other hand, *L. lenticularis* has testicular tissue interspersed throughout the ovotestes (Zaffagnini 1969; Scanabissi and Mondini 2002b). *Calalimnadia* presents a third configuration within the Limnadiidae: the testicular tissue is localized in the anterior

portion of the hermaphroditic gonad, directly behind the head. This is not unique positioning of the testicular tissue within the Spinicaudata; Brantner et al. (2013a) recently described a similar location of the testicular tissue in *C. gynecia*. However, it is more parsimonious to assume a convergence of the location of the testicular tissue in the Cyzicidae and Limnadiidae than to assume a common derivation of hermaphroditism for all of the Spinicaudata (i.e., linking the *C. gynecia* with *C. mahei*) that was then later lost by a whole suite of species but retained in *C. gynecia* and *C. mahei*. Instead, we believe that the anatomical evidence of the location of the testicular tissue is more likely to be reflective of three separate derivations of selfing-hermaphroditism within the Limnadiidae.

## Conclusions

We have described a fourth genus, *Calalimnadia*, in which selfing hermaphroditism is observed. The fact that hermaphroditism seems to have arisen at least three, possibly four times within two families of the Spinicaudata points toward underlying genetic mechanisms that provide lability of sperm production in females. With the current level of taxon and geographic sampling, we cannot definitively state that this fourth genus also constitutes a fourth derivation of hermaphroditism from dioecy, although anatomical evidence strongly suggests this conclusion. A resolution of the question of derivation of hermaphroditism within the Spinicaudata would likely arise from a greater taxon sampling within this suborder. Determining the breeding system of *Afrolimnadia alluaudi* would be helpful, as would a broader sampling of Africa to note whether *Calalimnadia* and *Afrolimnadia* are truly single-species genera. Additionally, continued histological surveying of the described androdioecious/hermaphroditic *Eulimnadia* would help to determine how rigid the location of testicular tissue is within this genus; if the *Eulimnadia* vary in the location of their testicular tissue, then a single derivation of hermaphroditism in the *Calalimnadia* + *Metalimnadia* + *Eulimnadia* would seem more feasible.

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