

## Ancient androdioecy in the freshwater crustacean *Eulimnadia*

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Among the variety of reproductive mechanisms exhibited by living systems, one permutation—androdioecy (mixtures of males and hermaphrodites)—is distinguished by its rarity. Models of mating system evolution predict that androdioecy should be a brief stage between hermaphroditism and dioecy (separate males and females), or vice versa. Herein we report evidence of widespread and ancient androdioecy in crustaceans in the genus *Eulimnadia*, based on observations of over 33 000 shrimp from 36 locations from every continent except Antarctica. Using phylogenetic, biogeographical and palaeontological evidence, we infer that androdioecy in *Eulimnadia* has persisted for 24–180 million years and has been maintained through multiple speciation events. These results suggest that androdioecy is a highly successful aspect of the life history of these freshwater crustaceans, and has persisted for orders of magnitude longer than predicted by current models of this rare breeding system.

**Keywords:** evolution of dioecy; breeding systems; conchostraca

### 1. INTRODUCTION

In our continuing attempts to understand the evolution of breeding systems, understanding the forces that select for a separation of the sexes (i.e. into pure males and pure females, termed dioecy), relative to a combination of the sexes (i.e. co-sexuals or hermaphrodites), has been a central theme (Charnov *et al.* 1976; Charlesworth & Charlesworth 1978; Charlesworth 1984; Schemske & Lande 1985; Jarne & Charlesworth 1993; Barrett 2002; Wolf & Takebayashi 2004). When selection favours a shift from one reproductive mode to the other (i.e. hermaphroditism to dioecy or vice versa), one of three temporary forms of reproduction is thought to accompany the transition: trioecy (mixtures of males, females and hermaphrodites), gynodioecy (mixtures of females and hermaphrodites) or androdioecy (mixtures of males and hermaphrodites). Of these, trioecy is thought to be highly unstable, and, thus, gynodioecy or androdioecy are predicted to be the two likely transitional reproductive modes between dioecy and hermaphroditism (Lloyd 1975; Charlesworth & Charlesworth 1978; Charlesworth 1984; Pannell 2002; Wolf & Takebayashi 2004). Gynodioecy is more common than androdioecy (Charlesworth 1984; Pannell 2002; Delph & Wolf 2005), which is expected by theoretical treatments of breeding system evolution (Lloyd 1975; Charlesworth 1984).

There are two categories of model for the persistence of androdioecy in species in which this breeding system has been documented (Pannell 2002): (i) androdioecy evolves

by the invasion of all-male individuals into an otherwise hermaphroditic species due to the selective advantages of being male-only, such as avoidance of inbreeding depression and increased mating advantages for male-only individuals (Lloyd 1975; Charlesworth 1984; Otto *et al.* 1993) or (ii) hermaphrodites that primarily devote energy to female function invade a dioecious population and replace females due to benefits for hermaphrodites of 'reproductive assurance' (the ability to produce offspring without a mate when mating opportunities are low; Pannell 1997; Wolf & Takebayashi 2004). Because the conditions for the stable maintenance of males with hermaphrodites are stringent in these models, androdioecy is predicted to be short-lived. The observations that true androdioecious species are sporadically distributed within the plant and animal kingdoms and that many plants described as androdioecious are actually 'cryptically dioecious' (i.e. hermaphrodites allocate virtually all their reproductive resources to female function, and are, thus, functionally female; Charlesworth 1984) appear to confirm this prediction (Pannell 2002). In fact, to date no multi-species, higher-level taxon (i.e. genus level or above) has been described as comprising only androdioecious species (Pannell 2002), again suggesting that androdioecy is a relatively recent and transient reproductive development in the various taxa in which it occurs.

One well described androdioecious system is found in the primitive crustacean class Branchiopoda, specifically in the clam shrimp *Eulimnadia texana* (Sassaman & Weeks 1993; Zucker *et al.* 1997; Weeks *et al.* 1999, 2000). Populations of these shrimp comprise males and

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self-compatible hermaphrodites, and population sex ratios range from 0 to 30% male (Weeks *et al.* 1999; Weeks & Zucker 1999). Interestingly, hermaphrodites in *E. texana* are of two genetically distinct types: 'amphigenic' hermaphrodites that produce hermaphrodites and males when selfed (in a 3 : 1 ratio) or outcrossed (in a 1 : 1 ratio), and 'monogenics' that produce only hermaphrodites either via outcrossing or selfing (Sassaman & Weeks 1993). This genetic sex-determining mechanism is unique to the Branchiopoda, only being described in *E. texana* (Sassaman & Weeks 1993), *Eulimnadia antlei* (Sassaman 1988), *Eulimnadia agassizii* (Weeks *et al.* 2005) and the notostracan *Triops newberryi* (Sassaman 1991). Evidence suggestive of this mechanism has also been reported in one other species in the branchiopod family Limnadiidae: *Limnadia lenticularis* (Tinti & Scanabissi 1996; Eder *et al.* 2000).

Sassaman (1995) suggested that four independent transitions from dioecy (the predominant reproductive mode) to unisexual reproduction has occurred in the clam shrimp, inferring that each transition was a rather recent evolutionary event. However, because of the ancient origin of the Branchiopoda, these 'recent' transitions could have occurred tens to hundreds of millions of years ago (Tasch 1987). One of these transitions is from dioecy to androdioecy, which Sassaman suggested occurred in the progenitor to both *Eulimnadia* and *Limnadia*. Chen & Shen (1981) suggest that *Eulimnadia* and *Limnadia* evolved from the extinct genus *Yunmenglimnadia* that existed in the Paleogene period (24–66 mya), while Tasch & Shaffer (1964) suggest that the genus *Eulimnadia* evolved in the Late Mesozoic era (*ca* 65 mya). If androdioecy is the predominant form of reproduction within the genus *Eulimnadia*, then this genus would represent the oldest clade of androdioecious organisms known (Pannell 2002), existing from 24 to 65 million years, which would indicate that androdioecy in these crustaceans is more stable than theory predicts (Lloyd 1975; Charlesworth 1984; Pannell 1997, 2002).

Herein we report data that demonstrate androdioecy in 11 additional limnadiid species in the genus *Eulimnadia*. Because the total number of species in this genus is currently debated (estimates range from 28 to 44), the individual species are geographically widespread, and the evolutionary relationships within the genus are not well understood (Belk 1989; Martin 1989; Sassaman 1995); we undertook a broad geographic sampling that included five continents and 10 countries (table 1). In this random sample of 13 (the 11 sampled herein as well as *E. texana* and *E. antlei*) of the species of *Eulimnadia*, we found evidence of androdioecy in each of these species and in no cases did we find evidence consistent with dioecy for any species. A phylogenetic assessment of the genus suggests that androdioecy evolved at or before the origination of this genus. We present evidence suggesting that this origination occurred 24–180 mya.

## 2. MATERIAL AND METHODS

To demonstrate that a species is functionally androdioecious, four criteria need to be met: (i) 'females' (here and throughout, we use this notation to underscore that 'females' are only morphologically typed, awaiting further evidence to

distinguish if they are true females or self-compatible hermaphrodites) must be able to gain fitness via both male and female function; (ii) 'females' produce offspring via a meiotic process; (iii) sexual type is genetically determined; and (iv) males actively persist in the wild via active fitness gain (e.g. as opposed to being in the population via mutation/selection balance, as in *Caenorhabditis elegans* (Stewart & Phillips 2002)). Because previous examinations have revealed that branchiopods have a unique form of androdioecy, in which two genotypic classes of hermaphrodites coexist with males (Sassaman 1988, 1989b; Sassaman & Weeks 1993), these four criteria can be collapsed into two (Weeks *et al.* 2005): (i) proof that 'females' can produce viable offspring when isolated from males; and (ii) proof that some of these 'females' produce offspring that are 100% 'female', while others produce 'females' and males in a 3 : 1 ratio, which would prove that these individuals are actually monogenic and amphigenic hermaphrodites, respectively (Sassaman & Weeks 1993). Data consistent with both criteria (i) and (ii) define the species as having the same androdioecious sex-determining mechanism as described in *E. texana* (Sassaman & Weeks 1993; Weeks *et al.* 2005).

Soil samples containing *Eulimnadia* cysts were collected from 10 locations: Western Australia (*E. feriensis*), Botswana (*E. africana*), Brazil (*E. brasiliensis*), Galapagos (*E. cylindrova*), India (*E. colombiensis*), Japan (*E. braueriana*), Mexico (*E. cylindrova*), Taiwan (*E. braueriana*), United States (*E. diversa*, *E. cylindrova*, *E. agassizii* and two new *Eulimnadia* species) and Venezuela (*E. colombiensis* and *E. follisimilis*) (table 1). Approximately 500 ml of these soil samples from each locality was placed in the bottom of 38 l aquaria and hydrated with deionized tap water. Aquaria were maintained under 'standard conditions' (Weeks *et al.* 1999). Under these conditions, nauplii hatch within 1–7 days and reach sexual maturity within 5–14 days post-hatching (depending on species).

Directly before sexual maturity, *ca* 50 'females' were isolated in 500 ml plastic cups containing approximately 5 ml of soil and filled with filtered water from the above hatching tanks. Isolated 'females' were allowed to lay eggs for *ca* 7 days and then were discarded. Eggs in the cups were then dried, the cups were sealed with lids, and were then placed in the dark for a minimum of 30 days.

After storing, the dried eggs were hydrated, using the methods outlined above. Hatching nauplii were transferred to 10 l plastic containers, and maintained under standard conditions. When the shrimp reached sexual maturity, sex ratios were determined on either all the surviving shrimp, or a subset if the total was much greater than 100 shrimp.

To facilitate the generation of DNA sequences for phylogenetic analyses, specimens of *Eulimnadia* were preserved in 95–100% ethanol when collected from the field while lab-reared specimens were frozen at  $-70^{\circ}\text{C}$ . Total DNA was isolated from 77 individual clam shrimp using the Qiagen DNeasy Plant Kit. A *ca* 1100 bp portion of 28S was PCR amplified using the D1F and D6R primers pairs (Park & O'Foighil 2000). The 28S amplicons yielded 954 bp of comparable sequence via cycle sequencing with the Perkin Elmer AmpliCycle Sequencing Kit using ddNTP–dNTP ratios optimized for automated sequencing. The amplicons were characterized by cycle sequencing analysis using dye-labelled sequencing primers with sequences identical to those of the PCR primers. The protocols for sequencing template purification and cycle sequencing of the fragments are as

Table 1. Population composition for all 10 *Eulimnadia* species. (*n*, total sample size; soil, sex ratios of shrimp reared from soil samples; amph, proportion of total hermaphrodites found to be amphigenic; offspring, proportion of males among offspring reared from amphigenic hermaphrodites.)

| species                | population    | soil     |                    | hermaphrodites |                    | offspring |                    |
|------------------------|---------------|----------|--------------------|----------------|--------------------|-----------|--------------------|
|                        |               | <i>n</i> | percentage of male | <i>n</i>       | percentage of amph | <i>n</i>  | percentage of male |
| <i>E. cylindrova</i>   | Galapagos     | 166      | 16.3               | 18             | 66.7               | 382       | 22.3               |
|                        | Mexico        | 37       | 27.0               | 13             | 46.2               | 338       | 30.2               |
|                        | Nebraska      | 34       | 17.6               | 13             | 53.8               | 1291      | 21.2               |
| <i>E. agassizii</i>    | Massachusetts | 176      | 0.0                | 20             | 0.0                |           |                    |
| <i>E. diversa</i>      | Arizona1      | 278      | 8.6                | 24             | 41.7               | 560       | 18.8               |
|                        | Arizona2      | 66       | 1.5                | 13             | 7.7                | 100       | 25.0               |
|                        | Arizona3      | 379      | 0.0                | 6              | 33.3               | 142       | 26.1               |
|                        | Arizona4      | 138      | 0.7                | 2              | 0.0                |           |                    |
| <i>E. braueriana</i>   | Japan1        | 100      | 12.0               | 48             | 58.3               | 2374      | 19.0               |
|                        | Japan2        | 216      | 19.4               | 20             | 65.0               | 1040      | 23.9               |
|                        | Taiwan        | 145      | 5.5                | 32             | 90.6               | 389       | 24.9               |
| <i>E. colombiensis</i> | India1        | 186      | 14.5               | 44             | 63.6               | 738       | 20.3               |
|                        | India2        | 13       | 0.0                | 3              | 0.0                |           |                    |
|                        | Venezuela     | 118      | 0.8                | 29             | 72.4               | 1528      | 20.7               |
| <i>E. follisimilis</i> | Venezuela     | 300      | 0.0                | 10             | 0.0                |           |                    |
| <i>E. africana</i>     | Botswana      | 23       | 30.4               | 10             | 80.0               | 1743      | 23.6               |
| <i>E. feriensis</i>    | Australia1    | 30       | 26.7               | 6              | 83.3               | 336       | 19.3               |
|                        | Australia2    | 27       | 0.0                | 8              | 0.0                |           |                    |
|                        | Australia3    | 89       | 14.6               | 9              | 55.6               | 65        | 21.5               |
|                        | Australia4    | 10       | 0.0                | 10             | 0.0                |           |                    |
|                        | Australia5    | 282      | 15.6               | 28             | 35.7               | 565       | 22.5               |
|                        | Australia6    | 9        | 33.3               | 1              | 0.0                |           |                    |
|                        | Australia7    | 148      | 27.7               | 21             | 47.6               | 920       | 26.2               |
|                        | Australia8    | 108      | 25.0               | 10             | 30.0               | 56        | 42.9               |
|                        | Australia9    | 84       | 29.8               | 4              | 50.0               | 111       | 8.1                |
|                        | Australia10   | 112      | 17.0               | 35             | 60.0               | 373       | 28.4               |
| <i>E. brasiliensis</i> | Brazil1       | 70       | 22.9               | 7              | 42.9               | 549       | 16.2               |
|                        | Brazil2       | 92       | 12.0               | 17             | 41.2               | 360       | 18.6               |
|                        | Brazil3       | 100      | 14.0               | 22             | 54.5               | 900       | 17.9               |
| <i>E. spp. A</i>       | Georgia       | 167      | 40.1               | 11             | 63.6               | 99        | 18.2               |
| <i>E. spp. B</i>       | Florida1      | 9        | 0.0                | 6              | 16.7               | 135       | 1.5                |
|                        | Florida2      | 133      | 1.5                | 4              | 0.0                |           |                    |
|                        | Illinois1     | 122      | 20.5               | 8              | 37.5               | 452       | 13.1               |
|                        | Illinois2     | 2        | 0.0                | 2              | 100.0              | 97        | 5.2                |
|                        | Indiana       | 141      | 20.6               | 18             | 66.7               | 269       | 20.4               |
|                        | Louisiana     | 27       | 0.0                | 13             | 0.0                |           |                    |
|                        | total         |          | 4137               | 12.3           | 545                | 49.2      | 15 912             |

presented in Folmer *et al.* (1994). These protocols include sequencing template purification in low-melting point agarose gels and cycle sequencing of both strands of each purified template using labelled primers. The separation of cycle sequencing reaction products was done in 3.7 and 5.5% polyacrylamide gels on LI-COR 4200L-2 and 4200S-2 automated DNA sequencers, respectively. The resulting sequences were aligned initially using ALIGNIR (ALIGNIR v. 2.0, LI-COR, Inc.), with subsequent refinement done manually using MACCLADE v. 4.0 (Maddison & Maddison 2000). All sequences generated for this project have been deposited in the GenBank database (see table 2 for accession numbers). The resulting 28S sequence matrix contained multiple 1–2 bp indels, but these caused no ambiguity in the alignment.

Phylogenetic analyses were conducted using the Bayesian inference (BI) algorithm in MRBAYES v. 3.0b4 (Huelsenbeck & Ronquist 2003). The GTR+I+G model, denoted as

appropriate for the 28S matrix by MODELTEST (v. 3.06; Posada & Crandall 1998), was used to correct for multiple hits in the BI analyses (10 chains, 5 million generations, 2 million generation burn-in). DNA sequences from *Metalimnadia*, the dioecious sister taxon to *Eulimnadia* (Hoeh *et al.* in press), were used to root the resulting *Eulimnadia* trees. The robustness of the BI trees was evaluated using posterior probabilities (PP). PAUP\* (v. 4.0b10; Swofford 2001) was used to select the tree topology with the highest log-likelihood score from among the trees saved during the BI analysis. This best tree topology was used by MACCLADE to conduct a parsimony-based breeding system character state optimization.

Distribution maps were created with ARCVIEW 8.1 GIS software (ESRI). Shape files were obtained from Earth System History GIS (Scotese 2001). Present day geographic coordinates were converted for use with historical continental maps using Point Tracker (Scotese 2002).

Table 2. GenBank accession numbers and locality information for specimens utilized in the phylogenetic analysis of 28S ribosomal DNA sequences.

| genus                              | species                           | spec. ID                 | 28S GenBank accession no. | locality                   |
|------------------------------------|-----------------------------------|--------------------------|---------------------------|----------------------------|
| <i>Eulimnadia</i>                  | <i>africana</i> (Brauer 1877)     | E.africanaZA W200        | DQ198196                  | South Africa               |
|                                    |                                   | E.africanaBW W261        | DQ198215                  | Botswana                   |
|                                    | <i>agassizii</i> (Packard 1874)   | E.agassiziiUS, MA NS58   | AY851430                  | United States: MA          |
|                                    |                                   | E.agassiziiUS, MA NS59   | AY851431                  | United States: MA          |
|                                    | <i>brasiliensis</i> (Sars 1902)   | E.brasiliensisVE NS106   | AY851415                  | Venezuela                  |
|                                    |                                   | E.brasiliensisBR W225    | DQ198203                  | Brazil                     |
|                                    | <i>braueriana</i> (Ishikawa 1895) | E.brauerianaBR W229      | DQ198204                  | Brazil                     |
|                                    |                                   | E.brauerianaJP NS40      | AY851425                  | Japan                      |
|                                    |                                   | E.brauerianaJP NS41      | AY851426                  | Japan                      |
|                                    | <i>cylindrova</i> (Belk 1989)     | E.brauerianaJP NS85      | AY851443                  | Japan                      |
|                                    |                                   | E.cylindrovaMX NS11      | AY851418                  | Mexico                     |
|                                    |                                   | E.cylindrovaMX NS16      | AY851422                  | Mexico                     |
|                                    |                                   | E.cylindrovaMX NS17      | AY851419                  | Mexico                     |
|                                    |                                   | E.cylindrovaEC NS65      | AY851432                  | Ecuador: Galapagos Islands |
|                                    |                                   | E.cylindrovaVE NS103     | AY851412                  | Venezuela                  |
|                                    |                                   | E.cylindrovaVE NS104     | AY851413                  | Venezuela                  |
|                                    |                                   | E.cylindrovaVE NS105     | AY851414                  | Venezuela                  |
|                                    |                                   | E.cylindrovaGP W165      | DQ198188                  | Guadeloupe                 |
|                                    |                                   | E.cylindrovaMQ W167      | DQ198189                  | Martinique                 |
|                                    |                                   | E.cylindrovaGP W177      | DQ198192                  | Guadeloupe                 |
|                                    |                                   | E.cylindrovaJP W204      | DQ198197                  | Japan                      |
|                                    |                                   | E.cylindrovaJP W205      | DQ198198                  | Japan                      |
|                                    |                                   | E.cylindrovaUS, NE W258  | DQ198213                  | United States: NE          |
|                                    |                                   | E.cylindrovaUS, NE W259  | DQ198214                  | United States: NE          |
|                                    |                                   | E.cylindrovaUS, NM NS9   | AY851444                  | United States: NM          |
|                                    |                                   | E.cylindrovaJP NS79      | AY851440                  | Japan                      |
|                                    |                                   | E.cylindrovaJP NS80      | AY851442                  | Japan                      |
|                                    |                                   | E.cylindrovaUS, NM NS35  | AY851424                  | United States: NM          |
|                                    |                                   | <i>dahli</i> (Sars 1896) | E.dahliAU W188            | DQ198194                   |
|                                    | E.dahliAU W189                    |                          | DQ198195                  | Australia                  |
|                                    | <i>diversa</i> (Mattox 1937)      | E.diversaUS, AZ NS8      | AY851441                  | United States: AZ          |
|                                    |                                   | E.diversaUS, AZ NS22     | AY851420                  | United States: AZ          |
|                                    |                                   | E.diversaUS, AZ NS23     | AY851421                  | United States: AZ          |
|                                    |                                   | E.diversaMX NS66         | AY851433                  | Mexico                     |
|                                    | <i>feriensis</i> (Dakin 1914)     | E.diversaMX NS67         | AY851434                  | Mexico                     |
|                                    |                                   | E.feriensisAU W98        | DQ198173                  | Australia                  |
|                                    |                                   | E.feriensisAU W100       | DQ198174                  | Australia                  |
|                                    |                                   | E.feriensisAU W101       | DQ198175                  | Australia                  |
|                                    |                                   | E.feriensisAU W102       | DQ198176                  | Australia                  |
|                                    |                                   | E.feriensisAU W103       | DQ198177                  | Australia                  |
|                                    |                                   | E.feriensisAU W104       | DQ198178                  | Australia                  |
|                                    |                                   | E.feriensisAU W105       | DQ198179                  | Australia                  |
| E.feriensisAU W106                 |                                   | DQ198180                 | Australia                 |                            |
| E.feriensisAU W107                 |                                   | DQ198181                 | Australia                 |                            |
| E.feriensisAU W112                 |                                   | DQ198182                 | Australia                 |                            |
| E.feriensisAU W113                 |                                   | DQ198183                 | Australia                 |                            |
| E.feriensisAU W115                 |                                   | DQ198184                 | Australia                 |                            |
| E.feriensisAU W163                 |                                   | DQ198187                 | Australia                 |                            |
| E.feriensisAU W184                 |                                   | DQ198193                 | Australia                 |                            |
| E.feriensisAU W231                 |                                   | DQ198205                 | Australia                 |                            |
| E.feriensisAU W233                 |                                   | DQ198206                 | Australia                 |                            |
| E.feriensisAU W236                 |                                   | DQ198207                 | Australia                 |                            |
| E.feriensisAU W238                 |                                   | DQ198208                 | Australia                 |                            |
| E.feriensisAU W240                 |                                   | DQ198209                 | Australia                 |                            |
| E.feriensisAU W242                 | DQ198210                          | Australia                |                           |                            |
| E.feriensisAU W246                 | DQ198211                          | Australia                |                           |                            |
| <i>magdalensis</i> (Roessler 1990) | E.magdalensisVE NS99              | AY851445                 | Venezuela                 |                            |
|                                    | E.magdalensisVE NS100             | AY851411                 | Venezuela                 |                            |
|                                    | E.magdalensisVE NS107             | AY851416                 | Venezuela                 |                            |

(Continued.)

Table 2. (Continued.)

| genus               | species                      | spec. ID                   | 28S GenBank accession no. | locality                |
|---------------------|------------------------------|----------------------------|---------------------------|-------------------------|
|                     | <i>texana</i> (Packard 1871) | E.texanaUS, NM JT5         | AY851410                  | United States: NM       |
|                     |                              | E.texanaUS, NM NS5         | AY851429                  | United States: NM       |
|                     |                              | E.texanaUS, NM NS34        | AY851423                  | United States: NM       |
|                     |                              | E.texanaMX NS50            | AY851427                  | Mexico: Baja California |
|                     |                              | E.texanaMX NS51            | AY851428                  | Mexico: Baja California |
|                     |                              | E.texanaUS, NM NS70        | AY851435                  | United States: NM       |
|                     |                              | E.texanaUS, NM NS71        | AY851436                  | United States: NM       |
|                     |                              | E.texanaUS, NM NS72        | AY851437                  | United States: NM       |
|                     |                              | E.texanaUS, NM NS73        | AY851438                  | United States: NM       |
|                     | <i>Eulimnadia</i> sp. A      | Eulimnadia sp.A US,GA W170 | DQ198190                  | United States: GA       |
|                     |                              | Eulimnadia sp.A US,GA W209 | DQ198200                  | United States: GA       |
|                     |                              | Eulimnadia sp.A US,GA W252 | DQ198212                  | United States: GA       |
|                     | <i>Eulimnadia</i> sp. B      | Eulimnadia sp.B US,IN W132 | AY851455                  | United States: IN       |
|                     |                              | Eulimnadia sp.B US,FL W139 | DQ198185                  | United States: FL       |
|                     |                              | Eulimnadia sp.B US,FL W140 | DQ198186                  | United States: FL       |
|                     |                              | Eulimnadia sp.B US,MS W174 | DQ198191                  | United States: MS       |
|                     |                              | Eulimnadia sp.B US,IL W207 | DQ198199                  | United States: IL       |
|                     |                              | Eulimnadia sp.B US,IL W223 | DQ198202                  | United States: IL       |
| <i>Metalimnadia</i> | <i>Metalimnadia</i> sp.      | MetalimnadiaBR NS109       | AY851417                  | Brazil                  |
|                     |                              | MetalimnadiaBR W265        | DQ198216                  | Brazil                  |

### 3. RESULTS

A total of 4137 clam shrimp were reared from egg banks collected from soil from 36 separate populations: 27 (75%) had both male and 'female' individuals (termed polymorphic populations), while eight comprised strictly 'female' individuals (table 1). Sex ratios from these egg banks ranged from 0 to 40% male. Of these 4137 shrimp, a total of 1254 'females' were isolated for egg production in individual cups. Of those, 545 had viable offspring hatch and survive even though no males were available to fertilize their eggs. From these 545 isolation cups, 33 787 clam shrimp offspring were reared to adulthood. Clearly, because these 'females' produced viable offspring in the absence of males (satisfying criterion (i) above), these original 545 'females' were either asexual females (e.g. clonal) or self-compatible hermaphrodites.

To assess criterion (ii), we measured the sex ratios of the offspring produced by the 545 isolated females. Within the polymorphic populations, there were two categories of female: those that produced no males among their offspring (typical of monogenic hermaphrodites) and those that produced some proportion of males among their offspring (typical of amphigenic hermaphrodites). Out of the 467 total isolates from the polymorphic populations, 56% produced males and 'females' among their offspring. Of the 15 912 offspring derived from these male-producing isolates, male percentage ranged from 2 to 30%, with an average of 21% males (table 1). Although this is close to the idealized frequency of 25% males produced by selfing amphigenic hermaphrodites (Sassaman & Weeks 1993), with the large sample size, it is significantly different than this ideal ( $\chi^2=135$ ;  $p<0.0001$ ). A male proportion lower than 25% is expected due to the 5–30% higher mortality rate of *Eulimnadia* males relative to hermaphrodites (Sassaman & Weeks 1993; Zucker *et al.* 2001), and, thus, the current proportion of males is quite close to the proportion of males from amphigenic hermaphrodites in the originally

described androdioecious species, *E. texana* (23.6% males; Sassaman & Weeks 1993). Therefore, the 545 originally isolated 'females' are best explained by assuming that they are not asexual females, but rather are a combination of monogenic and amphigenic hermaphrodites (Sassaman & Weeks 1993).

The *Eulimnadia* breeding system character state optimization on the best 28S tree topology is presented in figure 1 along with PP nodal support values greater than 50%. Despite the lack of complete resolution in the 28S tree, the more inclusive clades are well supported (some PP values greater than 50% were omitted to simplify the presentation). A square at the tip of a terminal branch indicates that individual was sampled from a population with a known breeding system. Thus, the broad phylogenetic distribution of the androdioecious breeding system within the *Eulimnadia* individuals depicted in figure 1, combined with the absolute lack of evidence for dioecy within the genus, is consistent with the hypothesis that the ancestor of *Eulimnadia* had an androdioecious breeding system.

### 4. DISCUSSION

The proposed rarity of androdioecy has been confirmed by comparative analyses. In the first such analysis, Charlesworth (1984) suggested that all 17 plant species that had been described as androdioecious to the date of publication were actually cryptically dioecious, with the 'hermaphrodites' actually functioning as females (i.e. allocating essentially no energy to pollen production). This observation led Charlesworth to conclude that 'androdioecy is probably not an important phenomenon.' In the most extensive comparative analysis of androdioecy to date, Pannell (2002) outlines up to 50 plants and 10 animals that have been described as androdioecious. Most of these androdioecious species are poorly studied, with only 11 plants and 4 animals being well documented as having androdioecious breeding systems (Pannell 2002).

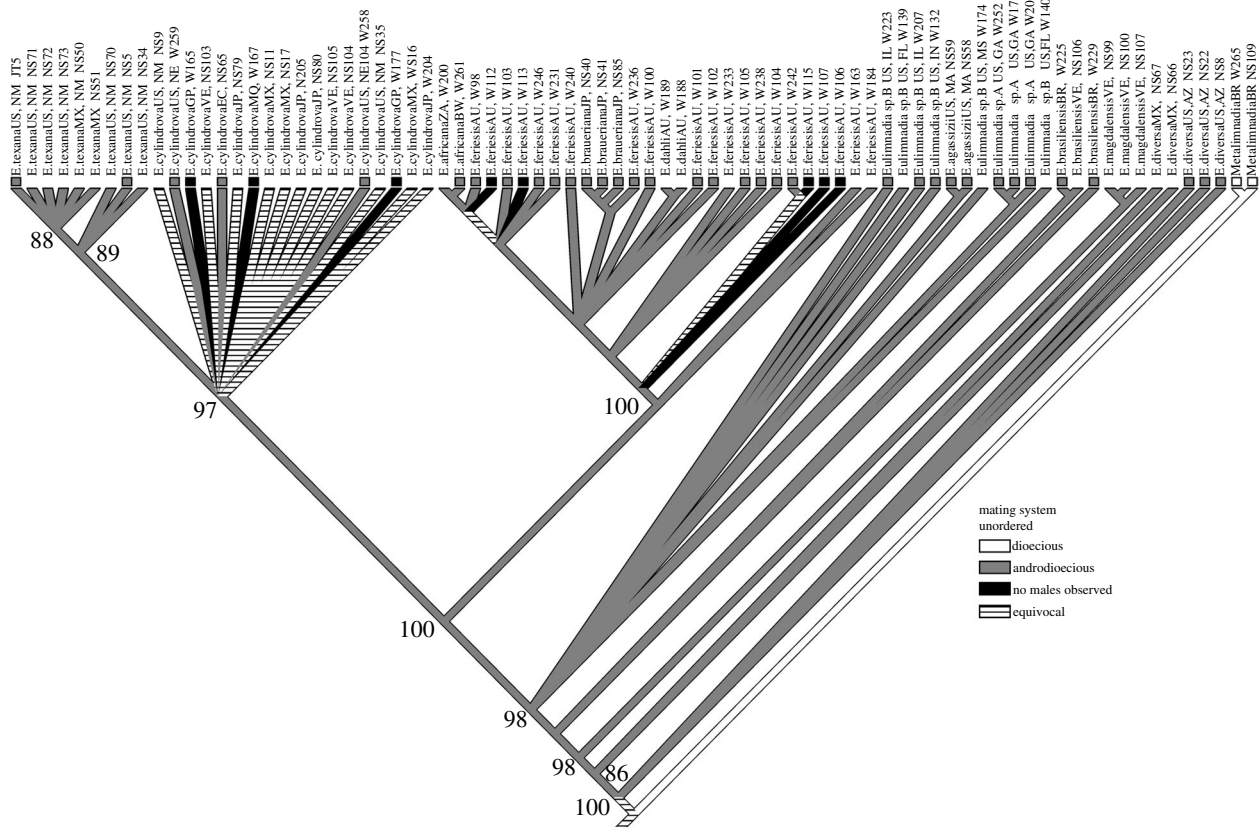


Figure 1. Breeding system character state optimization on the best 28S tree topology derived from Bayesian analysis of 77 individuals from 12 *Eulimnadia* species. A square at the tip of a terminal branch indicates that the individual was sampled from a population with a known breeding system. Branch colours (white, grey and black) denote breeding system type (dioecious, androdioecious and no males, respectively), which was either directly observed (those with squares) or predicted by MACCLADE. Hatched branches depict individuals for which MACCLADE could not unambiguously predict breeding system.

In addition to the 10 animal species outlined by Pannell, there may be up to an additional 17 species of barnacle that are androdioecious, in which larger hermaphrodites coexist with smaller, 'complemental' males (Weeks *et al.* in press). Thus, although androdioecy is not as rare as initially suggested (Charlesworth 1984), it is still uncommon among both plants and animals.

The predicted short-lived nature of androdioecy is also supported by the primarily terminal phylogenetic branches in which androdioecious species are found. Although up to 37 plant species in the family Oleaceae are morphologically androdioecious, only four of these appear to show functional androdioecy (Pannell 2002). Many of the remaining species exhibit close to 50% males, which is usually indicative of functional dioecy (Charlesworth 1984; Pannell 2002). There are several species of barnacle that have hermaphrodites and complemental males, including four in the genus *Balanus* (McLaughlin & Henry 1972). However, the genus *Balanus* has over 50 species (Pitombo 2004), so it is unclear whether these four species are all closely related (indicating a single derivation of androdioecy) or if they represent two or more instances of the evolution of complemental males. Therefore, to date, no higher taxon has been verified as being completely androdioecious, again consistent with the notion that androdioecy is a short-lived, transitory breeding system.

In the current study, the predominance of androdioecy in the genus *Eulimnadia* indicates that the precursor to this clam shrimp genus is most likely to have been androdioecious. The number of species in the genus *Eulimnadia* is

contentious, with estimates ranging from 28 (Straskraba 1964; Belk 1989) to 44 species (Brtek 1997). Because of these disagreements and the widespread nature of the genus, we specifically set out to examine a geographically broad sample of the genus. Herein we report data on nine of those species, plus two undescribed *Eulimnadia* species from the southeastern United States. Of these 11 species, nine showed definitive evidence of androdioecy (*cylindrova*, *diversa*, *braueriana*, *colombiensis*, *africana*, *feriensis*, *brasilensis* and the two undescribed species), while two had no males (*E. agassizii* and *E. follisimilis*), and, thus, criterion (ii) could not be assessed.

Although these latter two species did not show definitive evidence of androdioecy because of their lack of males, it is quite likely that they nevertheless have the same breeding system as the other 10 species. The lack of males in androdioecious clam shrimp is not uncommon. Among the 34 populations from the nine definitive androdioecious species studied herein, seven (21%) had 0% males (table 1), and, thus, were inferred to comprise monogenic hermaphrodites only. Monogenic-only populations are also common in *E. texana* (Sassaman 1989a). Because both *E. agassizii* and *E. follisimilis* were represented by a single population, it is quite likely that a larger sample of populations from both species would reveal them to comprise a polymorphic mix of amphigenic and monogenic hermaphrodites. Additionally, in a recent study (Weeks *et al.* 2005), *E. agassizii* has been shown to comprise monogenic hermaphrodites using anatomical and genetic evidence. Thus, 10 of the 11 species studied herein are

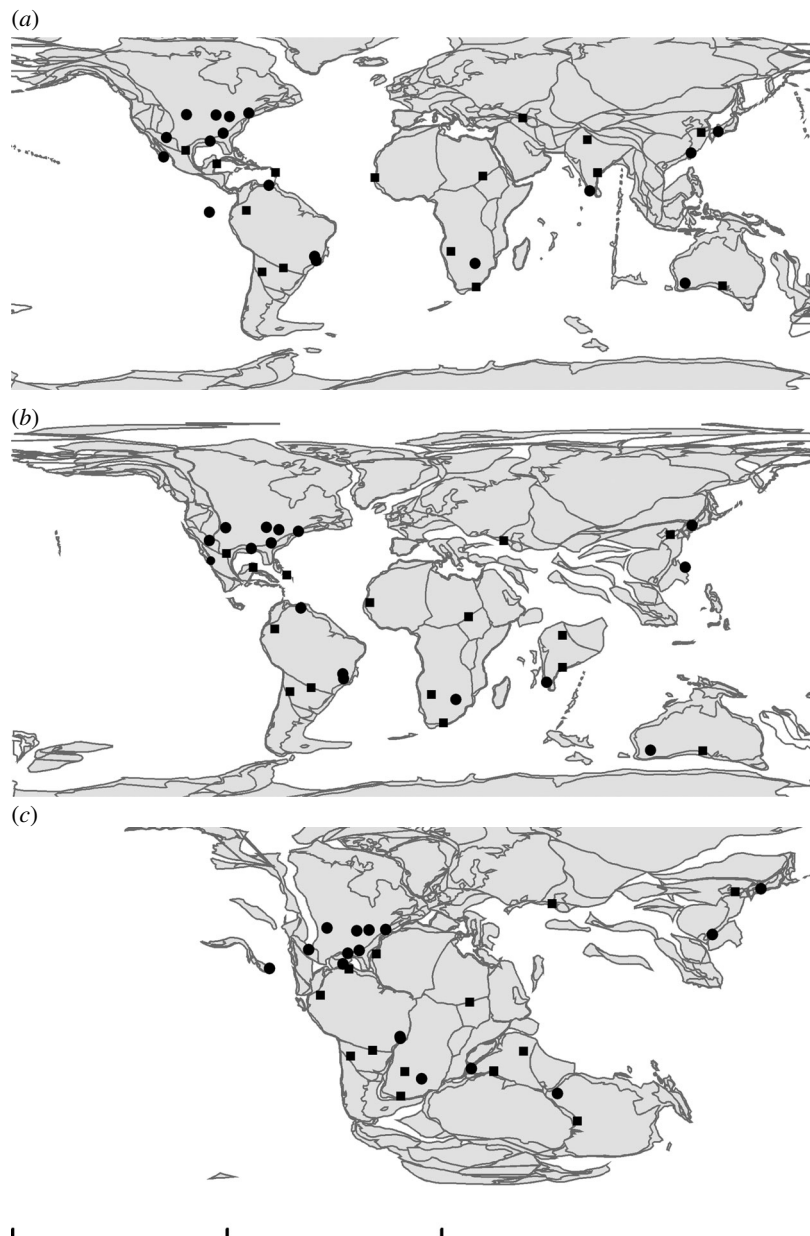


Figure 2. *Eulimnadia* distributions for the 11 species studied herein (circles) and the remaining known species in the genus (squares). (a). Current distributions. (b). Continental positions of the locations in (a) approximately 60 mya. (c). Continental positions of the locations in (a) when major continents were contiguous (ca 180 mya). These maps contain outlines of continental boundaries, shelf margins, major tectonic boundaries, active plate boundaries and seafloor spreading isochrones (Scotese 2001).

definitively androdioecious with the remaining (*E. follisimilis*) having patterns consistent with androdioecy.

In addition to the 11 species studied herein, two other *Eulimnadia* have been described as androdioecious. Sassaman & Weeks (1993) reported an extensive study of androdioecy in *E. texana*. Additionally, Sassaman (1988) reported that female *E. antlei* reared in isolation produced viable eggs, and when the resulting offspring were reared to adulthood, half the clutches 'were all-female; half consisted of mixtures of males and females in a 1 : 3 ratio' (pg. A135), which matches the average proportion of monogenics and amphigenics in the current study (table 1) as well as that previously reported for *E. texana* (Sassaman & Weeks 1993). Thus, *E. antlei* is also androdioecious because it meets criteria (i) and (ii) outlined above.

Therefore, in 12 of the 13 species so far examined in the genus *Eulimnadia*, offspring production matches the

expectations of the androdioecious breeding system first described in *E. texana*. Because these species probably represent a random subset of the 28–44 species of *Eulimnadia*, and are distributed across all continents that contain these shrimp, the most parsimonious explanation at present for the preponderance of this breeding system in *Eulimnadia* is that it arose at or before the origination of this genus. This contention is strongly supported by the phylogenetic analysis of 77 *Eulimnadia* individuals, which indicates both that *Eulimnadia* is monophyletic, and that the most basal species of the genus (*E. diversa*) is androdioecious (figure 1). Thus, the claim that androdioecy is the ancestral state for this genus is robust.

These data have two important ramifications. First, we can now claim that the minimum age of androdioecy in these shrimp must be at or before the origin of this genus, and, thus, if we can gauge the age of this taxon, we can

estimate the minimum persistence time of androdioecy in these crustaceans. Although clam shrimp have an excellent fossil record (Tasch 1987), the exact relationship of the extant limnadiid genera to fossil Limnadiidae is uncertain (Kobayashi 1972). Tasch & Shaffer (1964) suggest that the genus *Eulimnadia* evolved in the late Mesozoic era (*ca* 65 mya), while Chen & Shen (1981) suggest that *Eulimnadia* evolved from the extinct genus *Yunmenglimnadia* that existed in the Paleogene period (24–66 mya). Thus, from fossil evidence, the genus *Eulimnadia* is 24–66 million years old.

Another approach to ageing this genus is to examine the current species distribution and then to map this distribution onto ancient continental positions (Tasch 1987). The genus *Eulimnadia* has a global distribution, being reported from every continent except Antarctica (Baird 1849). This widespread distribution is not due to exceptional dispersal capabilities of these crustaceans (Tasch 1987), but is rather due to their ancient origin—estimated at *ca* 280 mya for the family Limnadiidae (Orr & Briggs 1999). Tasch convincingly argued that ‘dispersal of conchostracans eggs over land is well established for both living and fossil conchostracans...however, dispersal over oceanic distances by fortuitous winds is not’ (Tasch & Jones 1979). Tasch (Tasch 1971; Tasch & Jones 1979), therefore concludes that similar conchostracan fossils found on disparate continents are best explained by assuming the continents were contiguous at the time that the clam shrimp were fossilized.

Following the same logic regarding limited dispersal for extant distributions of *Eulimnadia*, we can use the various locations known to contain *Eulimnadia* and rotate the continents back to the time when these localities were contiguous (thus, allowing the dispersal of eggs among freshwater habitats). The current species of *Eulimnadia* were collected from India, Venezuela, the Galapagos, Mexico, Japan, Brazil, Botswana, Taiwan, Australia and the United States (figure 2a). We have also added known localities for many species of *Eulimnadia* not included in this study (figure 2a–c). The positions of the land masses 60 mya (corresponding to inferences of *Eulimnadia*’s origin from the fossil record; Tasch & Shaffer 1964; Chen & Shen 1981) still require large oceanic distances for eggs to disperse among continents (figure 2b). The last time period when the major land masses containing these locations were close enough for easy overland dispersal was *ca* 180 mya (figure 2c). Thus, if Tasch’s (Tasch 1971; Tasch & Jones 1979) suggested limitations on egg dispersal are correct, the precursor to the modern *Eulimnadia* was distributed across these five continents as much as 180 mya.

The second important ramification of our findings is that we now have evidence that androdioecy is not merely a phylogenetically short-lived breeding system (i.e. only found at the terminal portions of a phylogenetic tree). Because 12 of the 13 species so far examined in this multi-species genus are definitively androdioecious (and none show any hint of dioecy), *Eulimnadia* contradicts previous comparative analyses that suggest that androdioecy is not an important phenomenon (Charlesworth 1984). Apparently, these clam shrimp have retained androdioecy through multiple speciation events and across tens to hundreds of millions of years, suggesting that in this

genus, androdioecy has been a highly successful and important mode of reproduction.

Regardless of whether androdioecy arose 24 or 180 mya, it is clear that this form of reproduction has been quite stable in these shrimp for tens of millions of generations and has allowed for numerous speciation events within the *Eulimnadia*. This long-term stability is not predicted in any of the current models of the evolution of this breeding system (Lloyd 1975; Charlesworth 1984; Pannell 1997, 2002). Thus, the notion that androdioecy can only be a short-lived, transitional phase between hermaphroditism and dioecy (or vice versa) must be rejected. Just as any comprehensive theory of the evolution and maintenance of sexual reproduction must explain the long-lived, asexual bdelloid rotifers (Bell 1982; Welch & Meselson 2001; Barraclough *et al.* 2003), any truly comprehensive model seeking to delineate the benefits of separation of the sexes relative to hermaphroditism must now explain the long-lived coexistence of males with hermaphrodites in the *Eulimnadia* crustacea.

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