

# How ancient are ancient asexuals?

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Ancient asexual animal groups, such as bdelloid rotifers and darwinuloid ostracods, are excellent model organisms to study the effects of long-term asexuality. However, the absolute length of time that these groups have been fully asexual is mostly ignored. In the case of the darwinuloid ostracods, the fossil record shows that sexual reproduction disappeared almost completely after the end of Permian mass extinction (*ca.* 245 Myr ago), although several putative records of males from the Mesozoic obscure the exact time-frame of obligate asexuality in darwinuloids. Here, we re-examine the Mesozoic darwinuloid records, with regard to the reproductive mode of the assemblages. Three criteria to distinguish males in fossil populations (lack of brood pouch, position of muscle scars and size dimorphism) are used here to test for the presence of males in darwinuloid assemblages. A large, well-preserved assemblage of *Darwinula leguminella* (Forbes 1885) from the latest Jurassic (*ca.* 145 Myr ago) of England is found to be markedly variable in size and shape, but nevertheless turns out to be an all female assemblage. The exceptional preservation of the material also allows the re-assignment of this species to the extant darwinuloid genus *Alicenula*. All other putative dimorphic darwinuloid records from the Mesozoic are re-examined using the same criteria. The hypothesis that these assemblages represent bisexual populations is rejected for all post-Triassic (*ca.* 208 Myr ago) records.

**Keywords:** *Alicenula*; Darwinuloidea; Ostracoda; ancient asexuality; Jurassic; Cretaceous

## 1. INTRODUCTION

Darwinuloid ostracods and bdelloid rotifers have been called ‘ancient asexual scandals’ (Judson & Normark 1996), because they have survived for millions of years without sex and thus violate ruling evolutionary theory, which almost invariably predicts that fully asexual lineages are doomed to early extinction (Maynard Smith 1976). As such, ancient asexuals are considered excellent models to test the more than 20 hypotheses attempting to explain the paradoxical prevalence of sex in the animal and plant world (Kondrachov 1993; Butlin *et al.* 1998). Increasing attention is, therefore, devoted to the genetic signatures and evolutionary consequences of ancient asexuality (Mark Welch & Meselson (2000, 2001) and Arkhipova & Meselson (2000) on bdelloid rotifers; Schön *et al.* (1998) and Schön & Martens (1998, 2001, 2002, 2003) on darwinuloid ostracods).

When discussing ancient asexuality, however, one important issue is consistently overlooked: how ancient are ancient asexuals? Any molecular clock requires a real-time calibration, mostly against the fossil record. For bdelloid rotifers, the entire pre-Holocene fossil record is represented by a single piece of Dominican amber of Late Eocene to Early Oligocene age, 35–40 Myr ago, which contained 22 female rotifer specimens, at least 18 of which could be assigned with confidence to the Bdelloidea

(Poinar & Ricci 1992). This shows that bdelloids have existed for at least 40 Myr, but it tells us nothing about the total length of time that bdelloids have been fully asexual. Poinar & Ricci (1992) concluded that their fossil bdelloids reproduced parthenogenetically, but also admitted the possibility that certain lineages may have possessed amphimictic or cyclic modes of reproduction. The claims of a long absolute time-frame of parthenogenesis in bdelloids (e.g. Wuethrich 1998) may be exaggerated. However, molecular rate estimates (Mark Welch & Meselson 2001) extensively document relative time-frames in this group and confirm long-term asexuality.

Ostracods, however, generally have an extensively documented fossil record and this is certainly true for the Darwinuloidea. Three interpretations of the palaeontological data seem uncontested in the literature. First, the darwinuloids were most abundant and diverse in the Palaeozoic, especially in the Permian (Molostovskaya 2000), up to *ca.* 250 Myr ago (Bowring *et al.* 1998). More than 200 species in nine subfamilies and three families have been described, several of these in seemingly sexual populations (Abushik 1990). Second, most darwinuloids died out in the Early Mesozoic, leaving only a low diversity of species, all belonging to one family, the Darwinulidae, from about Mid-Triassic (*ca.* 230 Myr ago) up to the present day. Third, no sexual darwinulid populations have been found in this group for the last 65–100 Myr (Butlin & Griffiths 1993; Martens 1998).

However, this leaves a degree of uncertainty about the reproductive mode of darwinuloids for most of the Meso-

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zoic (i.e. between Mid-Triassic and Early Cretaceous). As the Recent darwinuloid fauna has its roots in the Mesozoic (Martens *et al.* 2003), this ambiguity about Mesozoic darwinuloids obscures the exact age and origin of the Cenozoic lineages.

Here, we present analyses of an extensive, well-preserved fossil assemblage of *Darwinula leguminella* (Forbes 1885) from the Jurassic, and of published records of other (Triassic to Early Cretaceous) putative examples of sexual dimorphism, which permit us to clarify some of the obscurity related to taxonomy and biology of Mesozoic darwinuloids.

## 2. MATERIAL AND METHODS

### (a) *Material*

Several hundreds of valves and carapaces of *D. leguminella* were obtained from a single sample of grey mudstone, collected from the Worbarrow Tout Member (Lulworth Formation) of the Purbeck Limestone Group (stratigraphical terminology of Westhead & Mather (1996)) on the east side of Lulworth Cove, Dorset, UK (latitude 50°37'00" N, longitude 2°14'30" W; National Grid Reference SY 828797). The sampled horizon, with a thickness of *ca.* 0.25 m, lies within the lower part of the *Cypridea granulosa* Subzone (*Theriosynoecum forbesi* Biozone) of Horne (1995), below the Cinder Bed Member that is sometimes taken to represent the Jurassic–Cretaceous boundary (but see Allen & Wimbledon (1991) for a discussion) and is therefore most probably of latest Jurassic age. The assemblage also contained the age-diagnostic cypridoidea ostracod *Cypridea granulosa granulosa* (J. de C. Sowerby 1836).

### (b) *Methods*

The mudstone sample was broken into small (centimetre sized) pieces and dried, then disaggregated by soaking in 10% hydrogen peroxide for a few hours, followed by washing through a series of sieves with mesh sizes ranging from 2 mm to 63 µm. The fraction retained on the 63 µm mesh was dried and ostracods were picked out under a binocular microscope. Illustrations and measurements of valves were made using scanning electron microscopy (SEM). The illustrated and measured specimens are deposited in the Mesozoic Invertebrates (M.I.) collections of the Royal Belgian Institute of Natural Sciences.

### (c) *Criteria for the recognition of darwinuloid males*

A combination of three morphological criteria for the recognition of darwinuloid males in the fossil record is used here for the first time.

#### (i) *Size distribution*

In most non-marine ostracods, males are either smaller (e.g. Cyprididae) or larger (e.g. Limnocytherinae) than females. In cases where sexual size dimorphism exists, a scatter plot of valve length-to-height (*L* : *H*) measurements will generate distinct clusters of points. The *L* and *H* values of 100 specimens were thus measured using SEM (accuracy ± 1 µm). The *L* and *H* values of each carapace were measured three times from a right lateral position. During a series of measurements, the *L* and *H* values of each carapace were measured once, so that each couple of measurements required repositioning of the carapace. This way, variability introduced by position of the specimen as well

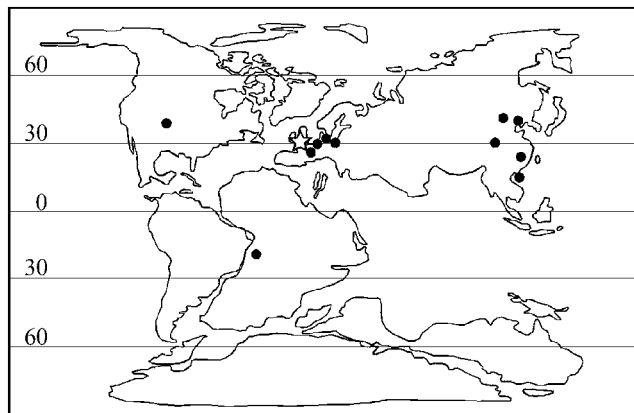


Figure 1. Palaeogeographical world map for the Early Cretaceous showing the known distribution of *Alicenula leguminella*. The species has been recorded (as *Darwinula leguminella*) from the Late Jurassic–Early Cretaceous period of England (Anderson & Bazley 1971; Anderson 1985 and herein), Germany (Martin 1940), Poland (Bielecka & Stejn 1966; Stejn 1991), Bornholm (Denmark) (Christensen 1963), France (Colin & Oertli 1985), China (Geng 1979; Gou *et al.* 1986; Li 1988; Xu 1993; Cao 1999), the Congo (Grekoff 1957) and the USA (Swain 1999). Dots, published records; star, locality at Lulworth Cove.

as possible bias introduced by the microscope are captured in the standard deviation. Only well-preserved carapaces were chosen and 59 sets of measurements were retained after any distorted or damaged carapaces were eliminated from the matrix. The Kolmogorov–Smirnov test, corrected following Lilliefors (1967), was used to test the valve measurements for deviation from normality.

#### (ii) *Size of the brood chamber in the dorsal view*

All extant darwinuloids are brooders (eggs and the first two to three instars are retained in the carapace). In all but one genus (*Microdarwinula*), species have an externally visible brood chamber, which causes the maximum carapace width in the dorsal view to be situated well behind the mid-length. It is assumed that putative males, lacking these pouches, would have their greatest width situated close to or at the middle of the length of the carapace, and be relatively shorter (this should be evident in the *L* : *H* ratios).

#### (iii) *Position of the adductor muscle scars*

Ostracod valves are closed by adductor muscles attached to the approximate centre of each valve, the functionally most efficient position. In adult brooding ostracods (e.g. female darwinuloids) the posterior extension of the carapace to form the brood pouch results in a relatively more anterior position for the adductor muscles. Adductor muscle scars (AMS) positioned more anteriorly on the inside of the valves (arrowed in figure 3*j–o*) may thus be taken as a female characteristic in darwinuloids with males being indicated by a more central AMS.

Similar criteria are used to identify sexual populations of darwinuloids in the Palaeozoic (and the Early Triassic), although in those assemblages the situation is more complex with some families having additional putative sexual dimorphism in valve morphology and structures. Post-Mid-Triassic darwinuloids all belong to the extant family Darwinulidae in which only the three criteria described in this section are relevant.

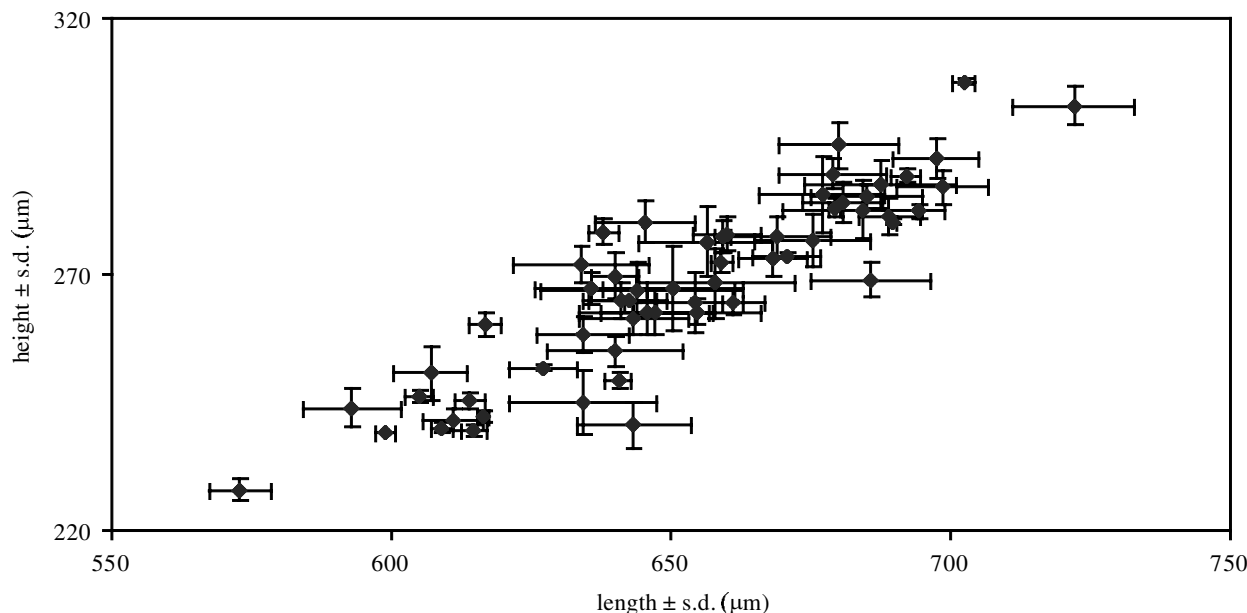


Figure 2. Scatter plot of the  $L:H$  measurements of 59 carapaces of *Alicenula leguminella* from the Purbeck of Lulworth Cove (UK), with crosses at each point representing the standard deviation from three replicate measurements.

### 3. RESULTS

#### (a) Taxonomic position of the Purbeck Limestone Group darwinuloids

The size and shape of the carapace (allowing for relatively wide variability), with the left valve (LV) overlapping the right valve (RV) on all sides, refers the present material to *D. leguminella*, a common Purbeck–Wealden (latest Jurassic–Early Cretaceous) species. The elongated valves, the absence of a keel in the RV and the presence of an antero-ventral and a posterior internal tooth in the LV all allow this species to be referred to the Recent genus *Alicenula*, Rossetti & Martens (1998). To our knowledge, this is the first confirmed Mesozoic record of this genus. The geographical and stratigraphical records of *Alicenula leguminella* are summarized in figure 1.

#### (b) Putative males in the Lulworth Cove assemblage?

When first investigating these specimens, we found some significantly smaller carapaces that were thought to be possible males, on the basis of comments by Christensen (1963), who reported putative males in *A. leguminella* from Purbeck deposits of Bornholm (Denmark). Because of the apparent interest in this discovery, our English assemblage was more thoroughly analysed.

##### (i) Size distribution

The results in figure 2 show a relatively wide range ( $L = ca. 570\text{--}725\ \mu\text{m}$ ;  $H = 220\text{--}310\ \mu\text{m}$ ), with most Lulworth Cove specimens falling between  $600\text{--}700\ \mu\text{m}$  for  $L$  and  $220\text{--}300\ \mu\text{m}$  for  $H$ . There are no distinctive clusters, which could either indicate the presence of males and females or even of adult and penultimate juvenile stage (A-1) females (Kolmogorov–Smirnov test for  $L = 0.075$  (d.f. = 58, lower limit of actual significance = 0.200), for  $H = 0.078$  (d.f. = 58; lower limit of actual signifi-

cance = 0.200)). We can thus conclude that the population is likely to be Gaussian and the smallest and largest specimens originally noticed thus constitute the extremes of a wide range of variability.

##### (ii) Size of brood pouch

All Lulworth Cove carapaces investigated, including the smallest specimens, show the greatest width being situated well behind the middle (visible in the dorsal view of figure 3*p–r* and the ventral view of figure 3*s–u*). All specimens thus have a clearly visible brood pouch. The scatter plot of the  $L:H$  measurements (figure 2) also shows that there are no large differences in the  $L:H$  ratios.

##### (iii) Muscle scars

In all of the valves from Lulworth Cove that did show the position of the AMS, even in the smallest ones (figure 3*l,o*) the AMS were situated well in front of the mid-length.

### 4. DISCUSSION

#### (a) Geographical and stratigraphical distribution of *Alicenula leguminella*

The known stratigraphical range of *A. leguminella* from the Purbeck–Wealden of England is latest Jurassic (Tithonian) to Mid–Early Cretaceous (Barremian) (Anderson 1985; Horne 1995). The age range of the Purbeck–Wealden of England thus lies between 146 and 125 Myr (Harland *et al.* 1989) and 141–119 Myr (Bralower *et al.* 1990), giving a total duration of between 21 and 22 Myr (see the discussion in Allen & Wimbledon (1991)) for the existence of *A. leguminella*. Thus far, *Darwinula stevensoni* (the most common extant darwinulid) was, together with *Microdarwinula zimmeri*, the longest living ancient asexual animal species (Straub 1952) with a documented stratigraphical range of 20–25 Myr. *A. leguminella* now equals this age.

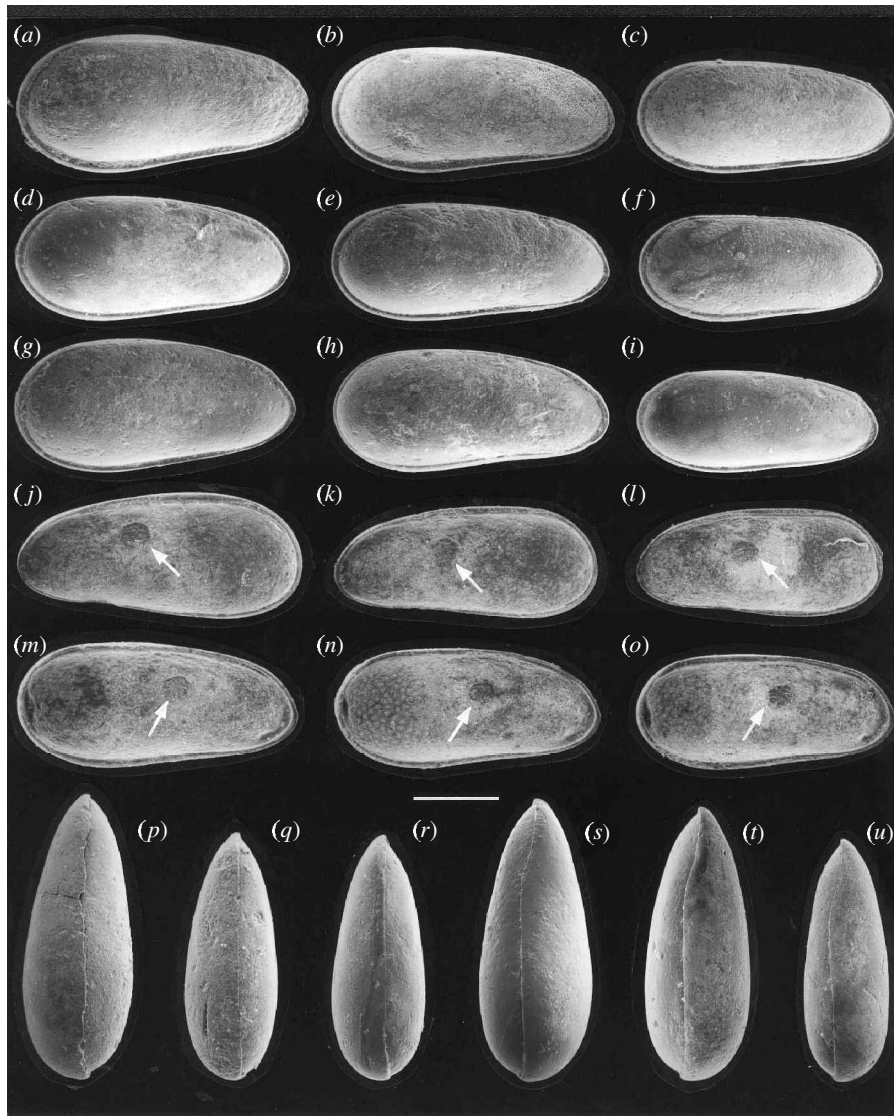


Figure 3. *Alicenula leguminella*, Lulworth Cove. (a–i) Carapaces, right lateral view (MI 10 860–10 868); (j–l) RVs, internal view (MI 10 869–10 871); (m–o) LVs, internal view (MI 10 872–10 874); (p–r) carapaces, dorsal view (MI 10 875–10 877); (s–u): carapaces, ventral view (MI 10 878–10 880). Scale bar, 200  $\mu$ m.

Published records (figure 1) seem to testify to a very broad geographical and stratigraphical range for the species. *A. leguminella* was widespread during the Early Cretaceous, at least in the Northern Hemisphere. Moreover the species was rather common, indicating a high ecological tolerance. The Recent *D. stevensoni* is both cosmopolitan and ubiquitous and it has recently been demonstrated (Van Doninck *et al.* 2002) that the general purpose genotype (GPG) in this species is most probably closely linked to its long-term asexuality. *A. leguminella* could be the Mesozoic ecological equivalent of *D. stevensoni*, its long stratigraphical range representing ample time for the development of a GPG.

The positive identification of *Alicenula* on the basis of valve characteristics allows the determination of a minimum age for the genus of ca. 145 Myr. This pushes the time of divergence between the extant darwinuloid genera back at least 50 Myr earlier than previous estimates, and strengthens the claim that both morphological and molecular evolution was very slow in the darwinuloid lineages (Rossetti & Martens 1998; Schön *et al.* 1998).

#### (b) Reliability of the three criteria to identify gender

For all three criteria, distinction between males and A-1 (female) juveniles could be difficult, as features related to the female brood pouch only develop during the final moult. Last-instar (A-1) juveniles will thus resemble males in valve shape and position of AMS and if males were to be smaller than adult females, also in size. The criteria used will thus have asymmetric power to test for the presence of males and only the absence of males will be unequivocally demonstrated.

Furthermore, fossil assemblages almost never represent a single population and generation; a bed a few centimetres thick is more likely to contain a time-averaged assemblage of valves and carapaces accumulated over hundreds of generations, depending on such factors as sedimentation rate and degree of bioturbation (Griffiths & Evans 1992). Fossil assemblages could thus comprise valves of ostracods that matured in different environmental conditions, which can lead to significant size differences between adults. It is well known that, for example,

Table 1. Records of putative males of Mesozoic darwinuloids.

species	reference	males reported?	based on				country	strata
			size	shape	AMS	figured		
<i>Alicenula leguminella</i>	Christensen (1963)	Y	+	+/-	-	-	Denmark	Late Jurassic–Early Cretaceous
<i>A. leguminella</i>	Xu (1993)	N	-	+	-	+	China	Early Cretaceous
<i>A. leguminella</i>	Cao (1999)	N	-	+	-	+	China	Early Cretaceous
<i>Darwinula sarytirmenensis</i>	Zheng (1995)	N	+	+	-	+	China	Mid-Jurassic
<i>D. cf. major</i>	Urosevic (1979)	Y	+	-	-	-	Yugoslavia	Late Triassic
<i>D. globosa</i>	Styk (1976)	Y	-	?	-	-	Poland	Triassic

temperature and salinity can significantly influence size and shape of ostracod valves (Martens 1985). Large series of specimens are needed to distinguish between size dimorphism and continuous variation and male records based on few specimens should thus not be accepted.

The visibility of the brood pouch strongly depends on how tightly the valves can close. In species of all darwinuloid genera (except *Darwinula s.s.*), valves have marginal structures such as internal teeth (mostly in the LV, see figure 3*m-o*) and an external posterior keel (in the RV of *Vestalenula*). These structures will limit the degree to which one valve can telescope into the other and will prevent the valves from closing too much and thus crushing eggs and juveniles. For example, in cases where the LV overlaps the RV (which is the case for most darwinuloids), the larger LV will have internal teeth, preventing the RV from entering beyond that point. As *Alicenula* has such internal teeth in the LV, the valves cannot be closed to the extent that the brood pouch becomes obscured, even after the death of the animal and the decay of its soft parts. In *D. stevensoni*, however, such structures are absent and non-ovigerous females indeed close their valves to the extent that the brooding space is no longer externally visible. This has at least once resulted, possibly, in the misidentification of a Recent specimen as a male (Brady & Norman 1889; but see Rossetti & Martens 1996).

### (c) Males in Mesozoic darwinuloids?

According to all three criteria the large darwinuloid assemblage from Lulworth Cove consists exclusively of females of *A. leguminella*. All specimens had clear brood pouches; where visible, all AMS were situated towards the front of the valves and no statistically significant size dimorphism could be detected in the assemblage. Rather, there is a large, normally distributed variability in the valve size. This suggests that the assemblage indeed resulted from the accumulation of a large number of generations, in which phenotypic variation may have been introduced by environment during (post-) embryonic ontogeny. Rossetti & Martens (1996) found a similar variability in size of valves in extant populations of *D. stevensoni* along a latitudinal gradient from Finland to South Africa.

There are several published examples of supposed dimorphism in Mesozoic darwinuloids (table 1). Some authors have specifically reported the presence of such putative males (Christensen 1963; Styk 1976; Urosevic 1979), others have illustrated supposed morphological dimorphism in size and/or shape, but did not explicitly

link this to the presence of males (Xu 1993; Zheng 1995; Cao 1999). All these records of putative males are highly doubtful, for various reasons as follows.

- (i) All studies are based on very limited material, e.g. Xu (1993) illustrated only two carapaces.
- (ii) Christensen (1963) also worked on *A. leguminella* of similar age to the material reported on here. We interpret the supposed size dimorphism reported by him in much the same way as our own, namely as extremes of a continuous variability. The shape difference reported by him does not become apparent from his *L:H* plot. He most probably simply observed the fact that shorter specimens are also narrower.
- (iii) None of the authors applied all three criteria used in the present study, in some papers no evidence for the presence of males is given at all (Styk 1976).
- (iv) In a number of cases (Urosevic 1979; Zheng 1995), the observed size dimorphism could very well be owing to the fact that authors were dealing with multi-species assemblages. Originally, Urosevic (1979) identified *Darwinula cf. oblonga*, *D. aff. major* and *D. cf. globosa*, but following his editor's opinion (N. Krstic, unpublished data) he illustrated them as 'male?', 'female?' and 'juvenile?' of *Darwinula cf. major*. The many other papers on Mesozoic darwinuloids (Kempff 1980, 1997) unequivocally reported on all-female populations.

In conclusion, none of the Mesozoic records of putative darwinuloid males are convincing, while certainly the post-Triassic records (*ca.* 208 Myr ago) can all be rejected based on the lack of conclusive morphological evidence. Obligate asexuality in Darwinuloidea therefore spans at least 200 Myr ago. The reported slow molecular (Schön *et al.* 1998) and morphological (Rossetti & Martens 1998) evolution of asexual darwinuloids is further corroborated by the fossil record as the Recent genus *Alicenula* is now known to have existed for at least 145 Myr.

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