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Phylogenetic evidence for a single long-lived clade of crustacean cyclic parthenogens and its implications for the evolution of sex

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The short-term advantages of sexual reproduction are unclear, but the existence of groups that are capable of producing either meiotic or ameiotic eggs (cyclic parthenogenesis, CP) might indicate that short-term advantages to sex exist. Alternatively, CP might be an unstable transitory stage between asexuality and sex, or a phylogenetically favoured life cycle (i.e. clade selection). The extensive knowledge of breeding systems and population genetics in branchiopod crustaceans makes them a useful group to test phylogenetic predictions of these hypotheses. Several proponents favour the hypothesis that CP has evolved multiple times in five orders of branchiopod crustaceans. We inferred the first robust branchiopod phylogeny from nuclear rRNA sequence (small-subunit and large-subunit), morphology, and complex rRNA stem–loop structures to assess the phylogenetic distribution of cyclic parthenogenesis. The sequence-based, structural rRNA and total evidence phylogenies are concordant and suggest that cyclic parthenogenesis arose once in the branchiopods, that this clade is long-lived (at least since the Permian), and that it has radiated extensively into nearly every aqueous habitat without reverting to strict sexuality and only rarely transforming to strict asexuality. These results are consistent with the clade selection hypothesis but inconsistent with the predictions of the hypothesis that CP is a transitory stage that leads to strict sexual reproduction. The evidence also indicates that clade selection for CP is a viable alternative explanation for the maintenance of sex in CP life cycles.

Keywords: parthenogenesis; rRNA; maintenance of sex; Crustacea; phylogeny

1. INTRODUCTION

For females that have much larger gametes than males, a lack of costly male production and the maintenance of successful genotypes are two clear short-term evolutionary advantages of asexual reproduction over sex. In contrast, the short-term evolutionary advantages of sex are unclear, leading to the problem of why sex is maintained. Life cycles that alternate between sexual and asexual reproduction have played a central role in discussions of this paradox. Williams (1966) and others (Barton & Charlesworth 1998) have argued that life cycles such as cyclic parthenogenesis (CP, an alternation of ameiotic and meiotic egg production) are difficult to explain without short-term or within-population advantages to sexual reproduction. This line of reasoning has led many researchers to focus on theory and experiments that assume sex is maintained because of short-term advantages. Nevertheless, the dismissal of long-term advantages to sex and cyclic parthenogenesis seems premature. For example, clade selection might favour cyclic parthenogens over either strict sexual or strict asexual reproduction. In this model, sex in cyclic parthenogens will be less of a 'choice' for a short-term advantage than of phylogenetic survival from long-term advantages linked to CP.

What might these long-term advantages be? Theoretical and empirical studies clearly show that by reproducing asexually most of the time with infrequent bouts of sex, CP can extend the short-term benefits of asexuality over the long-term (Lynch & Gabriel 1983; Lynch 1984; Hurst & Peck 1996; Michod 1998). This infrequent sexual phase of CP is sufficient to maintain a sexual population genetic structure (i.e. substantial genetic variation and linkage equilibrium (Lynch 1984)) thereby decreasing the long-term costs (such as an accumulation of harmful mutations and a lack of new gene combinations) and extinction rates of asexuality. Finally, CP might confer additional long-term advantages that neither asexuality nor sex alone imparts. Theoretical and empirical evidence indicate that facultatively sexual taxa are more susceptible to reticulate evolution (Ellstrand *et al.* 1996), rapid phenotypic change (Lynch 1984), non-geographic speciation (Mayr 1964) and enhanced dispersal (King & Murtaugh 1997). If clade selection is a foot race, then current theory suggests that asexuals are the sprinters and sexuals are the long-distance runners. Theoretically, CP enables a population to sprint most of the distance of the evolutionary marathon.

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It is important to emphasize that, although cycles of sexual and asexual reproduction are widespread in animals, most of these non-CP breeding systems lack the key characteristics that would impart the benefits of CP. For example, facultative selfing by hermaphroditic taxa strongly inhibits sexual population genetic structure and therefore some of the proposed advantages of sex (Hebert 1987*a*). Likewise, modular organisms that mix sexual with vegetative reproduction might be exposed to increased inbreeding and sustain additional costs from multicellular propagules, extra sex organs and a lack of independence for death and growth among conjoined clones (Charlesworth 1980).

The long-distance sprinter hypothesis assumes that mutation rates remain constant throughout evolution. However, Hurst & Peck (1996) have proposed an alternative 'one-way street' hypothesis that predicts that life cycles with sexual and asexual phases will be unstable over the long term because sex might increase the mutation rate. If sex improves the removal of harmful mutations and DNA repair is costly, then selection might favour decreased DNA repair in a cyclic parthenogen. This could lead to a positive feedback loop between sex and mutation rates. Eventually, the asexual phases would be eliminated, and, because complex breeding systems are difficult to regain once lost, strict sex should be the only stable breeding system. According to this model, clade selection will favour strict sexual breeding systems over either CP or asexuality.

If all things were equal then one might correctly infer the long-term advantages of a breeding system from current ecological success of taxa with a given life cycle. However, well-known constraints confound such an analysis. First there are constraints in switching between some breeding systems. For example, mammals are incapable of an asexual phase because genomic imprinting requires a paternal and a maternal set of genes. Similarly, evolving a breeding system that involves the production of two different kinds of egg (meiotic and ameiotic) might require a genetic *tour de force* that makes a transition to cyclic parthenogenesis difficult (Williams 1966; White 1973; Lynch 1984; Hebert 1987*a*). A prediction of this hypothesis is that phylogenetic transitions to cyclic parthenogenesis should be rare. Second, there can be additional historical constraints: the failure of cyclic parthenogens to replace thermophilic organisms with a different life cycle in hot springs does not necessarily mean that CP is an inferior breeding system. It more probably means that CP taxa have yet to evolve thermostable proteins.

Williams (1992) concluded that strong inferences can be made about long-term advantages or clade selection from the phylogenetic distributions of breeding systems. For instance, clade selection for cyclic parthenogenesis predicts that clades of cyclic parthenogens should have existed for evolutionarily significant periods of time (i.e. they should have undergone radiations and should possess numerous higher taxa). Derived lineages with differing breeding systems should be young and rare. Alternatively, if clade selection favours sexual reproduction, both cyclic and asexual reproduction should be found only in groups at the terminal branches of phylogenetic trees (i.e. the younger taxa).

Testing these predictions has been difficult because of a dearth of knowledge of both deeper phylogenies and breeding systems in most groups containing cyclic parthenogens (e.g. rotifers, trematodes and aphids). Branchiopod crustaceans (e.g. *Artemia*, *Triops* and *Daphnia*), however, are a promising group for investigations of the evolutionary history of cyclic parthenogens for several reasons. First, a majority of species have well-characterized breeding systems: species with unknown sexual stages are rare (Sassaman 1995) and population genetic studies have been performed on taxa from most of the 24 families (Hebert 1987*b*; Weider 1989; Boileau *et al.* 1992; Browne 1992; Berg & Garton 1994; Demelo & Hebert 1994; Muller & Seitz 1995; Sadler & Spitze 1995; Tinti & Scanabissi 1996; Davies *et al.* 1997; Hebert & Finston 1997; Sassaman *et al.* 1997). Second, the group is long-lived with several higher taxa (eight orders) and the earliest proposed fossils date back to the Upper Cambrian (Walossek 1993). Third, for branchiopods, absolute constraints on breeding system changes seem to be lacking and multiple transitions to both asexuality and cyclic parthenogenesis have been proposed (Woltereck 1919; Eriksson 1934; Mordukhai-Boltovski 1968; Starobogatov 1986; Fryer 1987; Dodson & Frey 1991; Martin 1992; Alonso 1996).

At present there is no robust molecular phylogeny for branchiopod crustaceans. Hanner & Fugate (1997) analysed taxa from four of eight currently recognized orders and reported poor resolution for deeper branches with mitochondrial DNA (mtDNA). Crease & Taylor (1998) identified branchiopod small-subunit (SSU) secondary structures with phylogenetic potential, but did not perform a phylogenetic analysis of the structures or the sequences. In this paper our aim was to establish the first robust branchiopod phylogeny to assess the distribution of cyclic parthenogenesis in crustaceans. We tested the hypothesis that cyclic parthenogenesis is not monophyletic (in agreement with the current taxonomy) in the eight orders of branchiopod crustaceans. Our approach included the use of a broad array of phylogenetic characters from morphology, rDNA sequence from the nuclear SSU and large-subunit (LSU)-rRNA genes, and rDNA secondary structure helices.

2. MATERIAL AND METHODS

(a) *Taxon sampling*

We extracted DNA from specimens belonging to all eight orders of Branchiopoda and 17 out of 18 recognized ingroup families. Only the Leptestheriidae was completely unrepresented, and this family is unambiguously a very closely related group to *Caenestheriella* (Olesen *et al.* 1996). Sample locations are listed in electronic Appendix A (www.pubs.royalsoc.ac.uk./publish/pro_bs/rpb1421.htm). For outgroups, we used representatives of four families of fairy shrimps, which are uncontroversially the most ancient extant order of branchiopods (Walossek 1993; Schram & Hof 1998).

(b) *Nuclear sequences*

We PCR-amplified three nuclear rDNA regions, gel-purified the products and directly sequenced them with the Amplitaq FS cycle sequencing kit and the ABI 377 genotyper. Primers for the 5′ end of the LSU rRNA gene (5′-GGGACTACCCCCT-

GAATTTAAGCAT-3′ and 5′-CCAGCTATCCTGAGG-GAAACTTCG-3′) amplified a region from nucleotide 3311 to 4434 in *Drosophila melanogaster* (M2107, M29800). These primers were modified for anostracans (5′)-TTGGCGACCCGCAATT-TAAGCAT-3′) and 5′)-TCCTGAGGGAAACTTCGGAGG-GAAC-3′).

SSU rDNA primers for V4 and V7 and their flanking regions are published (Crease & Colbourne 1998). Both strands of the LSU fragment were sequenced. All SSU sequences were from GenBank (X01723, AF014011, AF070093–AF070124 and AF070519) except for that from *Branchinecta paludosa*. The V4 primers failed for *Podon*, *Bosmina* and *Ilyocryptus*; we excluded them from sequence-based phylogenies.

(c) *Complex characters*

The coding of 56 morphological characters was extracted from Olesen (1998) and treated as unordered. Secondary structure stem characters are based on the structural hypotheses of Crease & Taylor (1998) and scored as present or absent. The absent character state is a one base-pair bulge, whereas stem structures were proposed on the basis of compensatory, free energy, and Monte-Carlo simulation evidence (Crease & Taylor 1998). Note that the secondary structure proposals themselves are meaningless for phylogenetics: it is critical only that we compared homologous regions and inserts. Four clearly homologous SSU rDNA helices (E23-1a, E23-C, 43-1 and 43-2) varied in their presence or absence among the branchiopod data set. Marked positional, structural and sequence identity across the taxa indicated homology for these rRNA helices. These regions are not part of the sequence analysis. The character states for V4 in *Podon*, *Ilyocryptus* and *Bosmina* were designated as missing for this analysis. All complex characters were weighted equally with DNA sequence characters in the total evidence analysis.

(d) *Alignment*

Sequences were assembled and initially aligned by using the Clustal V algorithm. Further alignment was based on conserved secondary structures. Following Swofford *et al.* (1996), we conservatively removed all ambiguous, hypervariable regions as well as sites with gaps in the core sequences. This left 1266 sites from the conserved cores of the rDNA gene family, 533 of these from the SSU. The entire sequence alignment and secondary structures are presented in electronic Appendix A. All phylogenetic analyses were performed with PAUP 4.0* (Swofford 1998) and Puzzle 4.0 (Strimmer & Von Haeseler 1996).

(e) *Signal and tests of fit to models*

Likelihood mapping (Strimmer & Von Haeseler 1997) provided an *a priori* indication of the signal in this alignment. This graphical tool plots likelihood quartets from a test set onto basins of attraction, of which there are three types: star-like, net-like and tree-like. Our data showed 92.4% in the tree-like areas and only 3.1% in the star-like areas, indicating a strong signal and potential for tree resolution. More relevant to our tests, when parthenogens and non-parthenogens are divided into groups, 98.0% of quartets map to a tree-like area. In the sequence analyses we minimized the effects of several potentially confounding factors (base compositional bias, rate heterogeneity and different transition–transversion rates) by estimating these parameters directly from the data and using only those that significantly improved the likelihood of the model (Goldman 1993). Rate heterogeneity parameters were added to the model both before and after changing the number of substitutional classes from two to six (Cunningham *et al.* 1998).

3. RESULTS

Comparative secondary structural analysis of SSU-rRNA sequences support cyclic parthenogen monophyly. Three derived stem–loop structures (E23-1a, E23-C and 43-1; figure 1) unite all of the cyclically parthenogenetic branchiopod taxa. An additional derived stem, 43-2, unites the four orders of cladocerans. These four structures or inserts are apparently absent from other known branchiopods and are probably absent from all other arthropods (Van de Peer *et al.* 1998). For the morphological analysis alone we found similar results to those of Olesen (1998). Maximum parsimony (MP) with branch-and-bound search found two optimal trees (CI = 0.775, RI = 0.898) of 102 steps. One supported the monophyly of cyclic parthenogens, the other placed *Cylestheria* within the clam shrimp order Spinicaudata. Both of these trees did, however, agree with the monophyly of the Cladocera (Anomopoda, Ctenopoda, Haplopoda and Onychopoda).

A hierarchical test of fit to a maximum likelihood (ML) model revealed that addition of the base composition ($\log L_0 = -6713.3$, $\log L_1 = -6708.7$, $-2\log\Lambda = 9.2$, d.f. = 3, $p < 0.025$), transition–transversion ratio ($\log L_0 = -6708.7$, $\log L_1 = -6519.9$, $-2\log\Lambda = 377.52$, d.f. = 1, $p < 0.001$) and six substitutional classes ($\log L_0 = -6519.9$, $\log L_1 = -6489.7$, $-2\log\Lambda = 60.4$, d.f. = 4, $p < 0.001$) significantly improved the model. A second series of hierarchical tests revealed that both percentage invariant sites ($\log L_0 = -6489.7$, $\log L_1 = -6083.4$, $-2\log\Lambda = 812.6$, d.f. = 1, $p < 0.001$) and the discrete gamma distribution (with four categories) improved the model ($\log L_0 = -6083.4$, $\log L_1 = -6051.1$, $-2\log\Lambda = 64.6$, d.f. = 1, $p < 0.001$). Adding rate heterogeneity parameters before changing to six substitutional categories resulted in a weaker model ($\log L_0 = -6109.2$). There was no significant base composition variation between taxa (test for homogeneity, $\chi^2 = 25.5$, d.f. = 72, $p > 0.99$).

The trees based on rRNA sequence provide strong evidence for the monophyly of taxa from five orders that are cyclic parthenogens. This association was insensitive to parameter variation and optimality criterion. The best trees under the optimality criteria of ML ($-\ln L = 6044.53$), minimum evolution (ME; one tree of length = 0.634) and MP (three trees of 1151 steps found with heuristic search and 40 random addition steps) each indicate monophyly. Support for CP monophyly was strong, with the bootstrap values after 1000 iterations for Cladocera (Anomopoda, Haplopoda, Onychopoda and Ctenopoda) and (Cladocera + *Cyclestheria*) at 93 and 100 (ME with Kimura two-parameter distance); less than 50 and 99 (MP) and ML quartet puzzle values of 96 and 99 (figure 2*a*). *A posteriori*, two-cluster likelihood mapping also strongly supported the monophyly of Cladocera (100% of quartets) and of *Cyclestheria*–Cladocera (97.9% of quartets). The internal branches (figure 2*a*) leading to Notostraca, Ctenopoda, Onychopoda, Anomopoda and Daphniidae were supported in all molecular analyses.

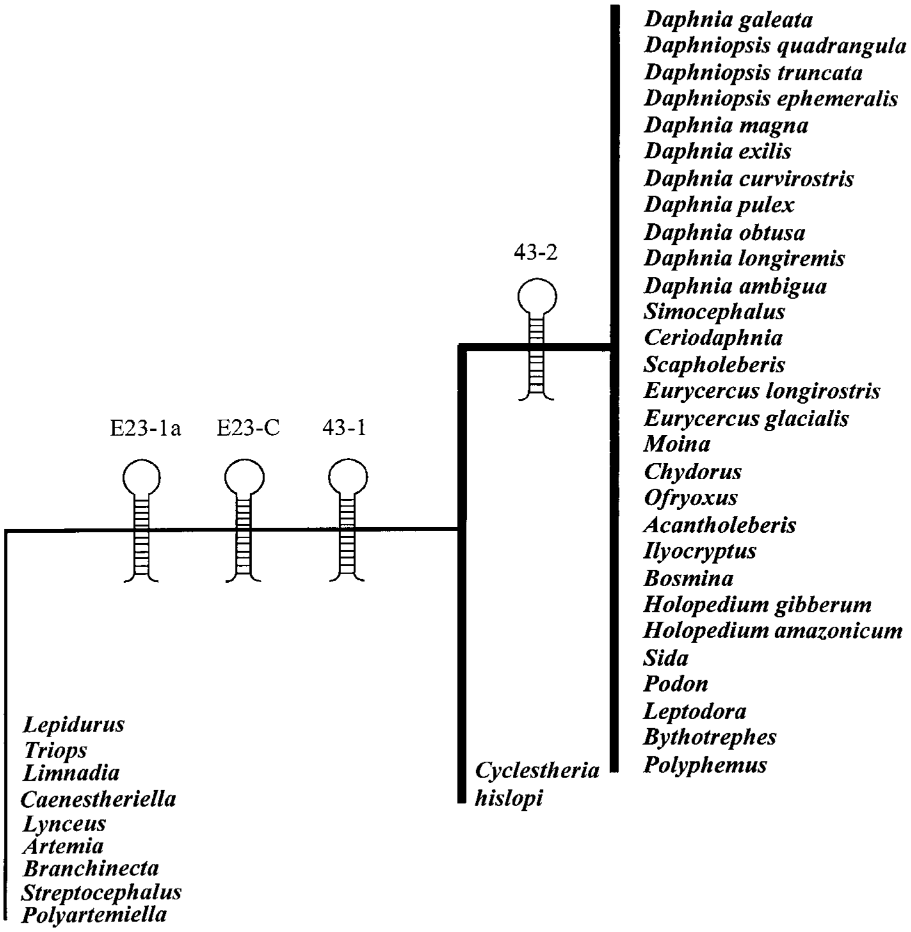

Figure 1. Phylogenetic tree based on the presence or absence of complex stem–loop structures in the nuclear SSU rRNA of branchiopod crustaceans. See electronic Appendix A for the secondary structure details. Bold lines indicate taxa with a cyclic parthenogen life cycle.

The total evidence MP tree (figure 2*b*) also strongly supported the monophyly of cyclic parthenogens with a bootstrap value of 92. The strict consensus trees from both weighted and unweighted searches with stepwise additions supported CP monophyly (unweighted: 49 trees of 976 steps, CI = 0.611, RI = 0.727; transversions weighted as two: four trees of 1265 steps, CI = 0.619, RI = 0.732). The best MP tree was significantly shorter, according to Wilcoxon signed-rank tests, than only one of two best trees constrained to be non-monophyletic with respect to *Cyclestheria*–Cladocera (length = 1274, $N = 21$, $T = 66$, $p = 0.0495$; length = 1274, $N = 25$, $T = 104$, $p = 0.0719$). The best tree was significantly shorter than all trees from a search constrained to be non-monophyletic for the four cyclic parthenogen orders (Cladocera) (length = 1289, $N = 23$–27; $T = 10.5$–37.5, $p < 0.0001$).

4. DISCUSSION

Our congruent molecular evidence strongly supports the monophyly of the cyclic parthenogens and the transitional nature of the clam shrimp family, Cyclestheriidae. The hypothesis of multiple transitions to CP emphasized the existence of divergent morphological and developmental differences (Fryer 1987). However, our molecular evidence identifies genetically based, complex homologous synapomorphies for the Cladocera and cyclic parthenogens (Cladocera + *Cyclestheria*). The unique form that branchiopod cyclic parthenogenesis takes also suggests a monophyletic origin of the group. The same female can produce both meiotic and ameiotic eggs (amphiteric parthenogenesis), whereas cyclically parthenogenetic females in other groups (e.g. aphids and most rotifers) produce either meiotic or ameiotic eggs, but not both. There are some taxa outside the *Cyclestheria*–Cladocera clade (e.g. *Artemia*, *Triops* and limnadiids) that have rare males, but there is a consensus that these groups are either obligate parthenogens or selfers (Browne 1992; Sassaman 1995; Sassaman *et al.* 1997). The derivation of cladocerans from a cyclestheriid ancestor is consistent with the fossil record because this family was present in the Permian when the first cladoceran fossils are recorded (Raymond 1946).

Our findings disagree with the predictions of the 'sex as a one-way street' hypothesis (see Hurst & Peck 1996). It is clear that the cyclic parthenogen clade is long-lived, having survived at least since the Permian (and probably much longer). The long-term stability in numerous habitat types and the lack of any known transition of CP to strict sex is inconsistent with the notion that a little bit of sex will eventually lead to only sexual reproduction. The stability of CP is unlikely to be due to constraints because the loss of a condition such as asexuality is probably an easy step (Williams 1992). Moreover, several waterfleas have sexual eggs that apparently differ little from their asexual eggs, suggesting an easy transition.

The results, however, are consistent with the predictions of the clade selection hypothesis for CP. The CP branchiopod clade is long-lived (probably before the Permian) with taxa from five orders and more than 77 genera that have radiated into most known aqueous habitats and lifestyles. There are herbivores, parasites, carnivores and

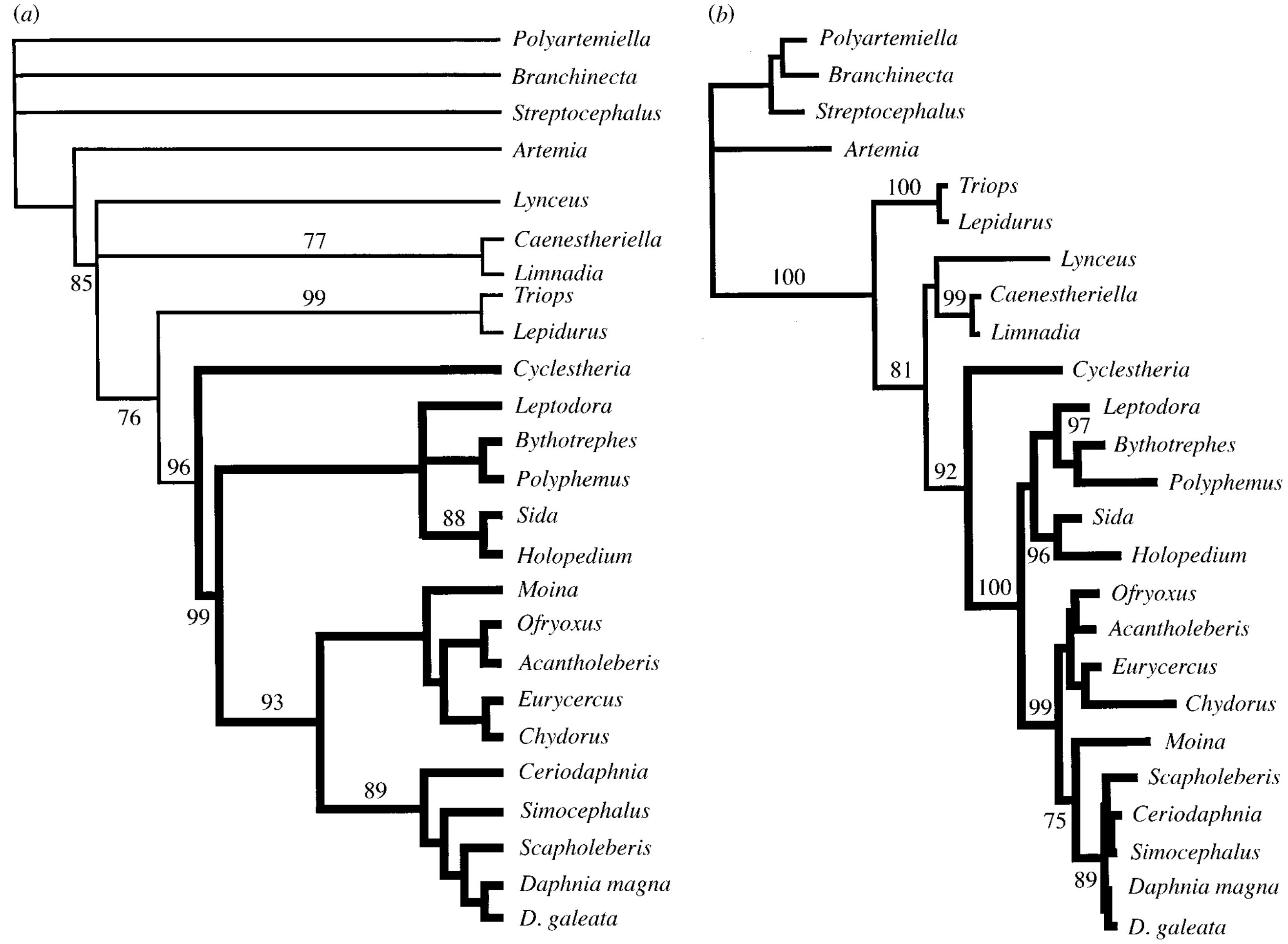

Figure 2. (*a*) Phylogenetic relations of branchiopod crustaceans inferred from variation in sequenced portions of SSU- and LSU-rDNAs. Internal branches with a bootstrap value of more than 70 are labelled with support values. Numbers indicate the quartet puzzle support value from 1000 iterations and an ML (likelihood = 6118.25) distance under the HKY model with invariable sites and four categories of gamma rate heterogeneity. Bold lines indicate taxa with a cyclic parthenogen life cycle. (*b*) Maximum parsimony tree based on the total evidence of morphology (Olesen 1998); nuclear rRNA and complex rRNA stem–loop structures (see electronic Appendix A). Bold lines indicate taxa with a cyclic parthenogen life cycle. Internal branches with a bootstrap value of more than 70 are labelled with support values from 1000 iterations of a heuristic search (stepwise addition). The tree shown is the MP tree with heuristic search algorithm (star decomposition method, tree length = 985).

detritivores living in habitats that range from moss in cloud forests to oceans. In contrast, the less diverse (43 genera) but much older sexual lineages of branchiopods are today largely restricted to one habitat type, temporary ponds. We note that clade selection works by differential extinction and speciation so that the number and the age of lineages are important but dominance in a particular habitat is uninformative. In addition, the stability of CP is unlikely to be a result of constraints to becoming an obligate asexual species because unrelated cladoceran groups (e.g. *Bosmina* and *Daphnia pulex*) have successfully made the transition to asexuality in the Anomopoda (Colbourne *et al.* 1997; Little *et al.* 1997).

If there is clade selection for CP, why is strict sex the most common breeding system? Although we cannot rule out other possibilities, our monophyly results are consistent with the old hypothesis that the rarity of CP is due to a combination of the difficulty of a germline in producing both meiotic and ameiotic eggs and historical contingency. Why this transition is difficult remains an elusive question, but the clam shrimp *Cyclestheria*, which might be the only extant member of a transition group to cyclic parthenogenesis, might provide clues. Hebert (1987*a*) proposed that the transition to CP from obligate sex occurred in the following sequence: development of two types of egg (diapause and immediate development), brooding of embryos, environmental sex determination, and meiosis suppression in one egg type. Although *Cyclestheria* does seem to possess these features (Roessler 1995), environmental sex determination needs to be confirmed. Roessler (1995) discovered that *Cyclestheria* lacks the full amphiteric cyclic parthenogenesis of cladocerans. That is, the same *Cyclestheria* female invariably fails to produce both meiotic and ameiotic offspring successfully. He did note, however, that parthenogenetic females do produce both meiotic and ameiotic eggs, but that these eggs are either reabsorbed or fail to develop. Thus, *Cyclestheria* might represent a transitional breeding-system stage that lacks the more complicated amphiteric cyclic parthenogenesis of cladocerans. Our results agree with those that suggest that a more detailed study of the *Cyclestheria* breeding system might provide insights into the origins of cyclic parthenogenesis.

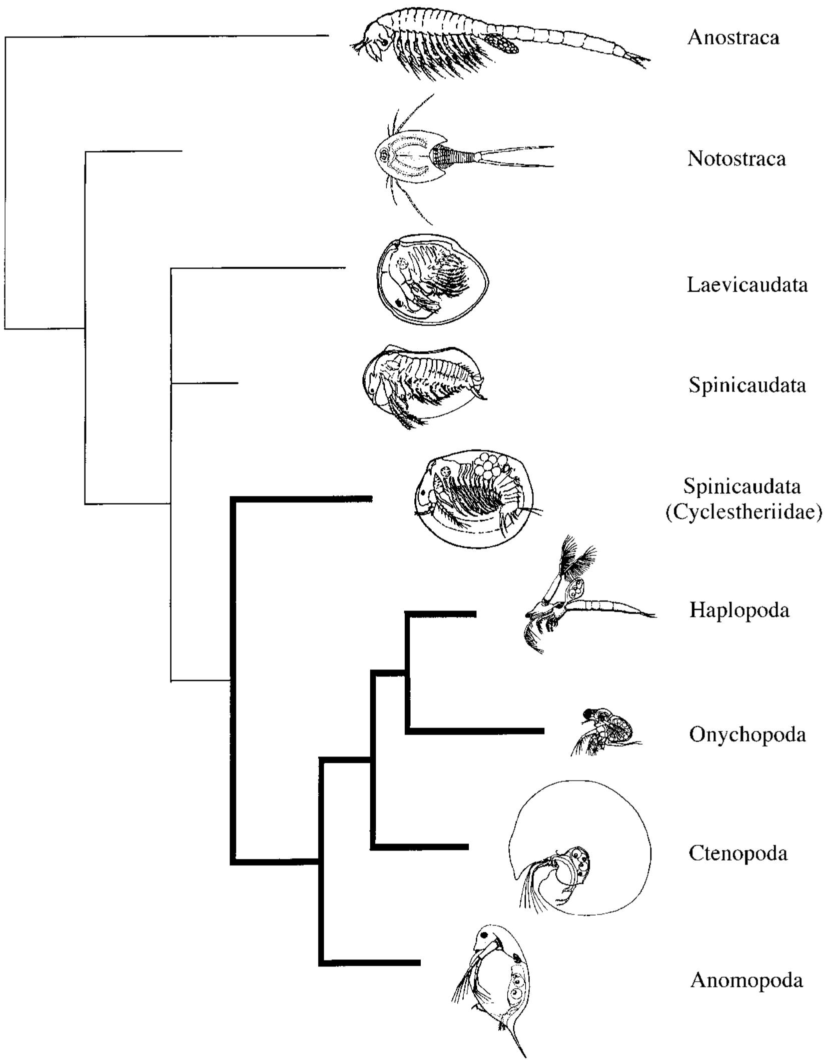

Figure 3. Summary of the ordinal relationships of branchiopod crustaceans based on the total evidence tree (figure 2). Note the paraphyly of the Spinicaudata. Bold lines indicate cyclic parthenogenetic taxa. At least two anomopod genera contain derived obligate asexual taxa (*Bosmina* and *Daphnia*). Representative mature females (not drawn to scale) are depicted for each order.

The present study used morphology, rRNA stem–loops and sequence characters to address the evolution of cyclic parthenogenesis. The results provide the first robust phylogenetic evidence that crustaceans from five orders with this breeding system are monophyletic (figure 3) and suggest that clade selection for CP is a viable alternative explanation to short-term advantages for the maintenance of sex. The generality of the evolution of crustacean cyclic parthenogenesis to other groups is unknown, and comparable knowledge (i.e. clear transition groups, robust phylogeny, evidence for antiquity of CP taxa and adequate breeding-system characterizations of most taxa) is not forthcoming. Nevertheless, if the same pattern becomes well established in other major groups then CP might more appropriately be termed 'the masterpiece of nature' than sexual reproduction. Moreover, there might be a need to establish why models based on the long-term benefits of sex (Nunney 1989) should be dismissed in favour of short-term benefit models.

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