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Evolution in the slow lane: molecular rates of evolution in sexual and asexual ostracods (Crustacea: Ostracoda)

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Parthenogenetic lineages within non-marine ostracods can occur either in mixed (with sexual and asexual females) or exclusively asexual taxa. The former mode of reproduction is associated with a high intraspecific diversity at all levels (genetic, morphological, ecological) and, at least in the Cypridoidea, with geographical parthenogenesis. Obligate asexuality is restricted to the Darwinuloidea, the strongest candidate for an ancient asexual animal group after the bdelloid rotifers, and is characterized by low diversity. We have compared rates of molecular evolution for the nuclear ITS1 region and the mitochondrial COI gene amongst the three major lineages of non-marine ostracods with sexual, mixed and asexual reproduction. Absolute rates of molecular evolution are low for both regions in the darwinulids. The slow-down of evolution in ITS1 that has been observed for *Darwinula stevensoni* (Brady & Robertson) apparently does not occur in other darwinulid species. ITS1 evolves more slowly than COI within non-marine ostracod families, including the darwinulids, but not between superfamilies. The ancient asexuals might have a higher relative substitution rate in ITS1, as would be expected from hypotheses that predict the accumulation of mutations in asexuals. However, the speed-up of ITS could also be ancient, for example through the stochastic loss of most lineages within the superfamily after the Permian–Triassic mass extinction. In this case, the difference in rate would have occurred independently from any effects of asexual reproduction. *Biological Journal of the Linnean Society*, 2003, **79**, 93–100.

ADDITIONAL KEYWORDS: accumulation of mutations – Cypridoidea – *Darwinula stevensoni* – Darwinuloidea – intraspecific diversity – mitochondrial COI genes – nuclear ITS region – substitution rate – superfamilies.

INTRODUCTION

PARTHENOGENESIS IN NON-MARINE OSTRACODS

Ostracoda are small (typically around 1 mm long), bivalved crustaceans that abound in most types of aquatic environments, both marine and non-marine. Apomictic parthenogenesis is common in non-marine ostracods (Bell, 1982), but its frequency differs between the three major extant lineages. All species in Darwinuloidea are fully asexual. In the Cytheroidea, a minority of species are fully asexual, whilst mixed reproduction is the most common reproductive mode in the Cypridoidea (Horne, Baltanas & Paris, 1998). Mixed reproduction in the Ostracoda does not involve cyclic parthenogenesis as in *Daphnia*, for example. Instead, two types of females occur, sexual and asexual. One would expect these to segregate into either sexual or all-female populations. However, mixed populations, in which males and both sexual and asexual females occur sympatrically, may be more common than originally thought (Chaplin, 1993). Parthenogenetic reproduction is less common in marine than in non-marine ostracods, although closer examination may reveal more asexual lineages in marine environments (Horne *et al.*, 1998). Partly for this reason, parthenogenesis is more prominent in the Cypridoidea

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than in the Cytheroidea (Martens, 1998), which comprises mainly marine lineages.

Ostracods offer an additional bonus for evolutionary research, as they have an excellent fossil record allowing more accurate time estimates for the onset of asexuality in the major lineages, which separated around 450 Myr ago (Martens, Horne & Griffiths, 1998). This is possible because most ostracods display sexual dimorphism in carapace shape and/or size: one can screen even very old assemblages for the presence or absence of males. Both Cytheroidea and Cypridoidea have had mixed reproduction for at least 150 Myr, while the Darwinuloidea have been exclusively asexual for at least 100 Myr (Griffiths & Horne, 1998; Martens, 1998) and most probably closer to 200 Myr (Martens *et al*. in press). The more recent fossil record also allows tests of specific hypotheses concerning the time-scales on which the costs and benefits of sexual reproduction operate. For example, Griffiths & Butlin (1995) analysed data from a set of Holocene lake cores from across Europe. They showed that asexual species have greater variability in abundance than species with mixed reproduction, as expected if they fail to evolve in response to environmental change, but they rejected the generally accepted view that asexual species colonize newly available habitats earlier than sexuals.

In Cyprididae, mixed reproduction generally leads to a segregated distribution of reproductive modes known as geographical parthenogenesis (Lynch, 1984). In Europe, this distribution pattern typically has sexual populations restricted to areas around the Mediterranean Sea, whereas asexual populations are found throughout Europe (Horne *et al.*, 1998). This pattern has been explained by different historical (Vandel, 1928) and ecological hypotheses (Löffler, 1990; Peck, Yearsley & Waxman, 1998). The more comprehensive 'Holocene stability hypothesis' (Horne & Martens, 1999), which combines new evidence on the Holocene climate with environmental factors, offers predictions which are more congruent with the observed distribution patterns, both Recent and Quaternary. The ready availability of distribution records, compiled in the NODE database (Non-Marine Ostracod Distribution in Europe, presently curated at Greenwich University, UK by D.J. Horne: http://www.gre.ac.uk/schools/nri/earth/ostracod/nodep1.htm) is another reason for using ostracods as model organisms.

Geographical parthenogenesis has previously been linked to the presence of a General Purpose Genotype (GPG) in clonal lineages of species with mixed reproduction (Lynch, 1984). A GPG is a genotype capable of producing either a single broadly tolerant phenotype or a plastic response allowing the lineage to survive in a wide range of environmental conditions. This reduces the need for the genetic diversity generated by sex. It has recently been shown that *Darwinula stevensoni* (Brady & Robertson), a darwinulid species that has persisted without sex for at least 25 Myr (Straub, 1952), indeed shows all the characteristics of a GPG (Van Doninck *et al*., 2002). It has been argued that a GPG can effectively only originate in lineages surviving over long time frames without any form of sex (K. Van Doninck, unpubl.).

Long-term persistence of asexuality through mixed reproduction

Asexual lineages within species with mixed reproduction survive through morphological, genetic and ecological diversification (Martens, 1998). For example, DNA sequence data of the cypridid species *Eucypris virens* (Jurine) revealed an exceptionally high intraspecific genetic diversity for both nuclear and mitochondrial DNA sequences (Schön *et al*., 2000). Values of intraspecific divergence are as high as interspecific levels reported for other animal groups. The high genetic diversity is also reflected in studies using allozyme polymorphisms (Rossi *et al*., 1998): no less than 211 multilocus genotypes were found in *E. virens*, the highest number ever reported for a non-marine ostracod. Phylogenetic reconstructions based on DNA sequences (Schön *et al*., 2000) have shown that asexuality in *E. virens* has originated several times independently, which makes dating of these asexual lineages all but impossible. The lack of consistent separation of asexual and sexual populations in the gene trees indicates that genetic exchange between these lineages is still ongoing, most likely by two processes: the continuous spin-off of asexual lineages from the ancestral sexual populations and hybridization between asexual females and males, either intra- or interspecifically. These mechanisms together could explain the exceptionally high genetic diversity of asexual clones in *E. virens* and other species (Butlin, Schön & Martens, 1998).

The genetic variability that is continuously formed through recombination in sexual lineages becomes frozen in the asexual spin-offs (Vrijenhoek, 1994) and ensures a variable gene pool among the clonal lineages. Ecological studies on different clonal lineages of *E. virens* (Otero *et al*., 1996) and *Heterocypris incongruens* (Ramdohr) have shown ecological specialization in some cases (Rossi & Menozzi, 1990, 1993; Rossi, Giordano & Menozzi, 1993), although the parallel existence of a few, more generally adapted clones in these species cannot be excluded (Martens, 1998). As a result, the asexual gene pool will show wide variability at all times and asexuality in these species with mixed reproduction will persist over long time

frames even though individual asexual lineages may go extinct.

The wide ecological variability is also matched by unusually high morphological variability (Yin, Geiger & Martens, 1999) and this has lead to considerable taxonomic confusion. Sexual and asexual populations of species with mixed reproduction have often been described as different species, for example the well-known North American *Limnocythere sappaensis* Staplin is actually a sexual population of the nearly cosmopolitan *Limnocythere inopinata* (Baird) (see discussion in Yin *et al.*, 1999). However, if species with mixed reproduction are indeed genetically inclusive clusters of sexual and asexual lineages, then taxonomic separation by reproductive mode becomes untenable. Moreover, since apparently sexual populations can in fact be mixed, comprising males and both types of females (Chaplin, 1993), the identity of the so-called sexual species is even more doubtful.

Low diversity in ancient asexual darwinulids

Schön *et al*. (1998) found low diversity for the nuclear ITS1 region and the mitochondrial COI gene within the exclusively asexual darwinulid species, *D. stevensoni*, and between *D. stevensoni* and two other members of this ancient asexual clade: *Microdarwinula zimmeri* (Menzel) and *Penthesilenula brasiliensis* (Pinto & Kotzian). For ITS1 in *D. stevensoni*, no genetic variability whatsoever was detected between populations from South Africa and Finland by direct sequencing (Schön *et al*., 1998). Sequence analyses of cloned ITS amplicons from *D. stevensoni* also revealed low genetic diversity within individuals (Gandolfi *et al*., 2001; Schön & Martens in press), while allozyme studies (Rossi *et al*., 1998) confirmed the low genetic diversity of *D. stevensoni*.

Morphological studies on the Darwinuloidea (Rossetti & Martens, 1996, 1998) show a number of small, but consistent, structural differences in valve and appendage anatomy between genera; small but stable differences, mostly in valve shape, between species in the same genus and low to very low (e.g. in *D. stevensoni*; Rossetti & Martens, 1996) intraspecific morphological variability. Differences between genera have accumulated over 100–200 million years, and the same might be true for at least some congeneric species. Phenotypic differences between the South African *Vestalenula molopoensis* (Martens & Rossetti) and the Australian *V. marmonieri* Rossetti & Martens are small, as these taxa basically differ in the size of the central muscle scars only (Martens & Rossetti, 2002). If these species started diverging from each other when Australia became separated from the rest of Gondwana, then this small morphological difference represents more than 100 million years of morphological evolution. This means that morphological evolution in the Darwinulidae has been exceptionally slow at three levels (generic, inter- and intraspecific; Schön *et al*., 1998).

Evolutionary rates in lineages with different reproductive modes

From this brief summary, it is clear that the two different classes of asexual taxa in non-marine ostracods, ancient obligate asexuals vs. parthenogenetic lineages with closely related sexual lineages, have very different evolutionary strategies leading to these patterns. Does this reflect different rates of molecular evolution? Both Muller's ratchet (Muller, 1964) and the mutation load hypothesis (Kondrashov, 1988) predict an increased rate of deleterious substitutions in asexual lineages. At neutral sites, the substitution rate is not expected to change, except perhaps as a response to selection for a reduction in mutation rate (Butlin, 2002). For mitochondrial DNA sequences which rarely, if ever, experience recombination, there is no reason to expect changes in substitution rates. This means that the ratio of nuclear to mitochondrial rates of molecular evolution can be used as an index of the effect of asexual reproduction where absolute rates cannot be calculated.

Here, we analyse a combination of previously published genetic data on *D. stevensoni* with additional sequences from other darwinulid species, thus comparing molecular evolution amongst all extant genera of the Darwinulidae. New dates from the fossil record are also taken into account. Evolutionary rates are compared to representatives with both sexual and mixed reproduction from the other two main lineages of non-marine ostracods. Calculations and tree constructions are mainly based on sequence data from the nuclear ITS1 region and the mitochondrial COI gene; sequencing of other nuclear genes such as *hsp82* is currently in progress. The ITS1 region is subject to some selective constraints (e.g. Schlötterer *et al*., 1994) and so mutation accumulation theories predict an increase in rate of substitution in asexual lineages. However, there is no easy way to distinguish selected from neutral sites, making tests for a change in rate less powerful than they would be for coding regions where at least a partial separation can be achieved.

MATERIAL AND METHODS

DNA was extracted from individual ostracods, and ITS1 and part of COI were amplified and sequenced as described in detail in Schön *et al*. (1998), Schön (2001), and Martens *et al*. (in press). Although Gandolfi *et al*. (2001) state that part of their sequenced ITS1 stretch of *D. stevensoni* belongs to the 5.8S and 18S genes, we

could not identify such conserved regions in our alignments of non-marine ostracods, which is why we analysed our sequenced region as a whole. All sequences of the current paper have been submitted to GenBank, accession numbers AF031284, AF031303-5, AF031308, AJ241480, AJ241482, AJ241486, AJ241522, AJ241539 and AJ534405–AJ534423. Sequences were edited by eye and were aligned with Clustal W (Thompson, Higgins & Gibson, 1994). Phylogenetic tree constructions and calculations of genetic distances were conducted with Tree Puzzle 5.0 (Schmidt *et al*., 2002) using a transition/transversion ratio estimated from the data sets and heterogeneous substitution rates (gamma with eight categories) with the substitution model of Hasegawa, Kishino & Yano (1985). Maximum likelihood trees were constructed both with and without the molecular clock assumption to test for equal rates of evolution using a likelihood ratio test. DNA sequences of COI were translated into amino acids with DAMBE (Xia, 2000), using the invertebrate mitochondrial code. Genetic distances on the amino acid level were estimated according to Adachi & Hasegawa (1996). Trees were constructed using both the amino acid and the nucleotide data set for COI. Indels in the ITS1 sequence set were not treated as informative characters in tree construction. Only one maximum likelihood tree is shown here: the ITS1 phylogeny for all non-marine ostracod species analysed, regardless of reproductive mode. All other trees are available from the first author on request.

Sequences from six species of ancient asexual Darwinuloidea were analysed: *D. stevensoni*, *P. brasiliensis*, *P. kohanga* (Rossetti *et al*.), *M. zimmeri*, *V. molopoensis* and *Alicenula inversa* (Martens & Rossetti). The Cypridoidea were represented by three species with mixed reproduction: *Eucypris virens*, *Prionocypris zenkeri* (Chyzer & Toth) and *Candellacypris aragonica* (Brehm & Margalef). The four representatives of the Cytheroidea comprised three sexual species: *Mesocyprideis irsacae* (Kiss), *Cyprideis torosa* (Jones), *Cytherissa sinistrodentata* Bronshtein, and one species with mixed reproduction (*L. inopinata*). GenBank sequences from other crustaceans were used as out-groups: *Daphnia lumholtzi* (Sars) (acc. nr. AF308974) for COI and *Marsupenaeus japonicus* (Bate) (acc. nr. AF253524) for ITS1.

RESULTS

Rates of molecular evolution in non-marine ostracods

The relationships among the non-marine ostracod species inferred following analyses of the two sets of sequences are consistent with their systematic positions and the placement of Darwinuloidea as the sister group to Cypridoidea, which in turn is consistent with interpretations of the fossil record (Fig. 1). The molecular clock assumption can be rejected for both loci (COI: $-2\Delta L = 181.5$, d.f. = 9, $P < 0.01$. ITS1: $-2\Delta L = 711$, d.f. = 9, $P < 0.001$, using in-group taxa common to both data sets only), indicating that the rate of molecular evolution is unequal among non-marine ostracods. For ITS1 (Fig. 1), there are rate deviations in the cyprid branches, but the most striking effect is the long basal branch of the darwinulid clade and the short branches within that clade. For ITS1, alignment between the superfamilies is extremely difficult and so the lengths of the basal branches are unreliable.

When the ratio of COI distances to ITS1 distances is compared (Table 1), a clear pattern emerges: ITS1 evolves much more rapidly, relative to COI, in branches between superfamilies than it does within superfamilies. The pattern is consistent with observations in other taxa (e.g. freshwater crayfish; Harris & Crandall, 2000) and mostly probably reflects periods of turnover among rDNA repeats that apparently cause bursts of sequence evolution. These bursts are more likely to be encountered in the long interfamilial branches.

For a simple relative rate test, average HKY85 distances were compared between the three superfamilies (Table 2). As Darwinuloidea are sister to the Cypridoidea in both ITS1 (Fig. 1) and COI trees (not shown),

Table 1. Ratios of COI distances to ITS1 distances. Figures are ratios of mean pair-wise HKY85 (Hasegawa *et al*., 1985) distances between species for taxa common to the two sequence data sets

	Number of comparisons	COI : ITS1 ratio
Within Cypridoidea	1	1.06
Within Cytheroidea	6	2.08
Within Darwinuloidea	10	1.83
Between superfamilies	38	0.44

Table 2. Relative rates of evolution in non-marine ostracods. Average genetic distances between superfamilies are given with standard deviations in brackets

	Genomic region	Mean genetic distance		
		Dar–Cyp	Dar–Cyt	Cyp–Cyt
HKY85	ITS1	2.4180 (0.2352)	2.8664 (0.3090)	1.7803 (0.0987)
HKY85	COI	0.4764 (0.2736)	0.7804 (0.3074)	0.8011 (0.3658)

Dar = Darwinuloidea; Cyp = Cypridoidea; Cyt = Cytheroidea.

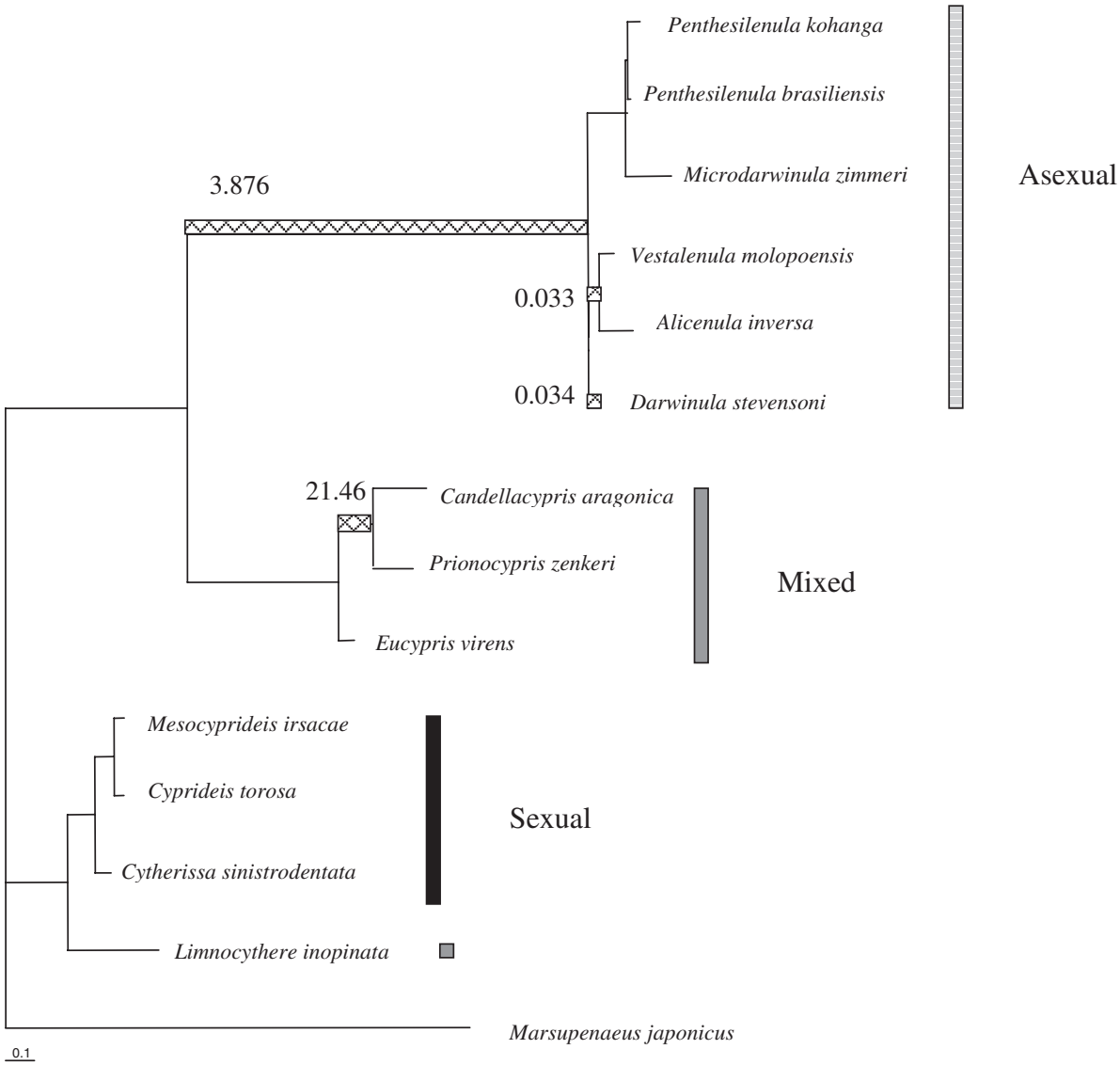

Figure 1. Maximum likelihood tree of ITS1 sequences from non-marine ostracods. The tree is constructed from HKY 85 distances with heterogeneous rates. ML tests for equal rates of evolution were conducted with the in-group dataset only. Gamma distribution parameter alpha of this data set is 3.45 ± 1.03 with and 2.61 ± 0.67 without out-group. The clock assumption is rejected with $-2\Delta L = 711$, d.f. = 9, $P < 0.001$. Hatched branches are those showing the greatest deviations in rate. For these branches, ratios of branch lengths without and with the clock are given. Other nodes show ratios of 0.133–1.605. Differently shaded bars indicate modes of reproduction.

their average distances to the Cytheroidea should be equal. The calculated distances fulfil this expectation for COI but not for ITS. Here, average distance between the Darwinuloidea and Cytheroidea is markedly greater than between Cypridoidea and Cytheroidea.

Variation in rate of molecular evolution amongst ancient asexual darwinulids

We repeated the sequence analysis for the asexual darwinulids only. In this case, COI nucleotide substitution rates do not depart significantly from the clock assumption ($-2\Delta L = 5.90$, d.f. = 3, $P > 0.1$), but ITS1 rates do ($-2\Delta L = 83.47$, d.f. = 4, $P < 0.01$). The branch leading to *M. zimmeri* shows the biggest rate deviation with a ratio of 2.659 for branch lengths without and with the clock assumption.

Absolute substitution rates can be estimated using evidence from the fossil record (as in Schön *et al.*, 1998, but with updated time estimates) together with maximum likelihood branch length estimates (Table 3). In this case, the branch to *M. zimmeri* again

Table 3. Molecular rates of evolution in Darwinulidae. Rates are based on dated notes from the maximum likelihood trees and are all given as HKY85 distances (Hasegawa *et al*., 1985) per million years. *A.* = *Alicenula*; *V.* = *Vestalenula*; *P.* = *Penthesilenula*; *M.* = *Microdarwinula*. N/A = not available

Species	Fossil age of lineage	ITS	COI
A. inversa from Vm/Ai node	145 Myr	0.00104	0.00107
V. molopoensis from Vm/Ai node	145 Myr	0.00035	0.00162
P. brasiliensis from Pb/Mz node	35–60 Myr	0.00043	0.0040–0.0068
P. kohanga from Pk/(Pb+Mz) node	35–60 Myr	0.00033–0.00056	N/A
Pk/Mz from Pk/(Pb+Mz) node	35–60 Myr	0.00224–0.00384	N/A
M. zimmeri from Pb/Mz node	25 Myr	0.0069	0.010

shows the highest rate. *Vestalenula* and *Penthesilenula* represent the other extreme. *Alicenula* also has a low rate of substitution based on a new fossil date of 200 Myr ago for the origin of this lineage (Martens *et al.* in press.) All of these rates are low compared with estimates for these loci in other invertebrate taxa (e.g. 0.01 for ITS1 in *Drosophila*, Schlötterer *et al*., 1994; 0.02 for COI in Crustacea, Knowlton *et al*., 1993).

DISCUSSION

These data tend to confirm earlier suggestions that overall evolutionary rates in the ancient asexual darwinulid ostracods are low compared to other invertebrates. The absolute rate estimates in Table 3 are about 10% of the rates observed in other taxa (Knowlton *et al*., 1993; Schlötterer *et al*., 1994). The exceptionally low level of ITS1 diversity in *D. stevensoni* (Schön *et al*., 1998; Gandolfi *et al*., 2001; Schön & Martens, in press) is not duplicated at the supraspecific level. Within *D. stevensoni*, ITS1 variability is much lower than would be expected from comparison with the diversity of mitochondrial COI sequences. Homogenizing mechanisms such as gene conversion (Butlin, 2000; Schön & Martens, in press) probably act in the ITS region, but also highly efficient DNA repair has been put forward as an explanation (Schön & Martens, 1998) for the exceptionally low genetic diversity in the nuclear genome of *D. stevensoni*. Because the differential pattern of ITS and COI diversity is not observed at a higher taxonomic level, it can not be associated with the ancient asexual darwinulid lineage as a whole. *Microdarwinula zimmeri* has a special position within the family, showing the highest rate of molecular evolution (Table 3) due to its large genetic distance to the other darwinulids. Phylogenetic reconstructions from both molecular and morphological data, together with fossil evidence, indicate that this species has split from its relatives quite recently (Martens *et al*. in press). Its exceptional position is reflected in its valve morphology – it is the only darwinulid without an externally visible brood pouch and has other derived features associated with its interstitial mode of life (Rossetti & Martens, 1998).

In order to determine whether or not their slow rate of molecular evolution is associated with the asexual mode of reproduction, it is necessary to compare darwinulids with other non-marine ostracods. Molecular divergence within the other two lineages should, ideally, be calibrated against real-time estimates but this requires further analysis of the fossil record.

Both nuclear and mitochondrial substitution rates appear to be low in darwinulids. Within the darwinulids, the ratios of COI to ITS1 divergence are similar to those in other non-marine ostracods with sexual or mixed reproduction. They are also similar to ratios in other invertebrates (the COI rate is approximately twice the ITS1 rate at low levels of divergence). This suggests generally slow evolutionary rates for all non-marine ostracods rather than a change of rate for nuclear loci associated with the origin of long-term asexuality in the darwinulids.

These conclusions are based on comparisons within superfamilies and so rely on relatively recent events. Inferences from basal branches are less reliable because of uncertainties in the alignment of ITS1 sequences and saturation of silent sites in the COI sequences. However, two observations are worthy of comment: the ratio of COI to ITS1 divergence is much lower between superfamilies and the basal branch of the darwinulids shows particularly rapid ITS1 evolution.

The pattern of low ITS divergence between closely related species, but much greater than expected divergence at higher taxonomic levels, has been observed in other taxa (e.g. freshwater crayfish; Harris & Crandall, 2000). One possible explanation is that occasional expansion in copy number of rare divergent repeat types causes periods of rapid change in sequence.

Unusually rapid ITS evolution in the basal branch leading to the darwinulids is suggested by the relative rate test (darwinulid–cytherid distance > cyprid–cytherid distance, Table 2) and by the clock vs. no-clock comparison (darwinulid branch 3.4 times longer than expected, Fig. 1). To the extent that ITS1 evolution is constrained by selection, this increase in rate could be attributed to mutation accumulation following the origin of asexuality. In the *hsp82* gene in bdelloid rotifers there may also be an increased rate of evolution in the basal branch even though this is not seen in comparisons between extant taxa (Mark Welch & Meselson, 2001; Butlin, 2002).

However, the independent history of the Darwinuloidea spans more than 350 Myr, whereas the lineage has only been exclusively asexual for about 200 Myr (Martens *et al*., in press). The speed up in ITS evolution might equally likely be an ancient event dating back to the Palaeozoic (>250 Myr) when Darwinuloidea were speciose and sexual reproduction was supposedly not uncommon. Therefore, it is impossible to decide now whether any acceleration of evolutionary rate was indeed associated with the transition to asexuality.

The Darwinuloidea as a superfamily also experienced a severe loss of lineages and species in the early Triassic (*c.* 200 Myr), after the Permian-Triassic (P-T) mass extinction. This stochastic loss of most lineages might have caused the apparent speed-up of ITS evolution in the only surviving lineage. The current results do not allow us to distinguish between the above hypotheses. In order to do so in the future, further analyses of the fossil record for the other two non-marine ostracod superfamilies (Cypridoidea and Cytheroidea) is required to provide more precise estimates of evolutionary rates and to assess the overall effect of the P-T mass extinction. Furthermore, comparisons of substitution rates in nuclear coding regions between the three non-marine ostracod lineages with different reproductive modes are needed to solve the paradox of 'ancient asexual scandals' (Judson & Normark, 1996).

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