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Slow molecular evolution in an ancient asexual ostracod

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Genetic variability of the non-marine ostracod species *Darwinula stevensoni* was estimated by sequencing part of the nuclear and the mitochondrial genome. As Darwinulidae are believed to be ancient asexuals, accumulation of mutations should have occurred, both between alleles within lineages and between lineages, during the millions of years of parthenogenetic reproduction. However, our sequence data show the opposite: no variability in the nuclear ITS1 region was observed within or among individuals of *D. stevensoni*, despite sampling a geographical range from Finland to South Africa. Lack of allelic divergence might be explained by concerted evolution of rDNA repeats. Homogeneity among individuals may be caused either by slow molecular evolution in ITS1 or by a recent selective sweep. Variability of mitochondrial cytochrome oxidase (COI) was similar to intraspecific levels in other invertebrates, thus weakening the latter hypothesis. Calibrating interspecific, genetic divergences among *D. stevensoni* and other Darwinulidae using their fossil record enabled us to estimate rates of molecular evolution. Both COI and ITS1 evolve half as fast, at most, in darwinulids as in other invertebrates, and molecular evolution has significantly slowed down in ITS1 of *D. stevensoni* relative to other darwinulids. A reduced ITS1 mutation rate might explain this inconsistency between nuclear and mitochondrial evolution in *D. stevensoni*.

Keywords: asexuality; ostracod; evolutionary rates; ITS1; COI

1. INTRODUCTION

Long-term apomictic reproduction will lead to the accumulation of genetic differences between clonal lineages and, since recombination is absent, between alleles within lineages (Judson & Normark 1996; Birky 1996). Thus, the extent of DNA sequence variability, and especially the maximum depth of branching among apomictic lineages, can be used to infer the duration of apomictic reproduction (Avise 1994) while sequence comparisons between alleles within individuals can be used to confirm that reproduction has been strictly apomictic (Judson & Normark 1996). These questions are of particular importance for lineages that have apparently existed without sexual reproduction for long periods of time because the accumulation of deleterious mutations through the operation of Muller's ratchet (Muller 1964) is expected to preclude their persistence (Hurst & Peck 1996; Kondrashov 1993; Maynard Smith 1989). However, the levels of divergence among lineages that can be observed in the present may be influenced by selective sweeps which, in strictly apomictic lineages, will homogenize genotypes at all loci, including the mitochondrial genome.

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Together with Bdelloid rotifers and the shrimp *Artemia salina* (Judson & Normark 1996), the ostracod family Darwinulidae is one of the best known and supported examples of an ancient asexual lineage (Butlin & Griffiths 1993; Judson & Normark 1996; Schön *et al.* 1996), having apparently reproduced asexually for more than 60 Ma. In such a case, the genetic consequences of apomictic reproduction should become extreme. The arguments against persistence without sex for extended periods led Judson & Normark (1996, following Maynard Smith 1986) to coin the term 'ancient asexual scandals'.

Darwinula stevensoni is a non-marine ostracod with a nearly world-wide distribution, living in a wide range of habitats (Griffiths & Butlin 1994). The species seems to have reproduced asexually for at least 25 Ma, maybe even longer (Butlin & Griffiths 1993; Schön *et al.* 1996), based on the absence of males. Apomixis has not been demonstrated directly but is the reproductive mode in other asexual ostracods (Havel & Hebert 1989). We have investigated genetic variability among *Darwinula stevensoni* individuals from 19 European and one South African sites, and genetic divergence from two other (also asexual) species of the Darwinulidae.

Unlike the nuclear genome, evolutionary divergence of mitochondrial DNA is not expected to be influenced by the reproductive mode since it is always inherited clonally. Therefore, parts of both the nuclear (ITS1 region) and the mitochondrial (cytochrome oxidase I) genomes of

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Darwinula stevensoni were amplified, sequenced and compared. ITS1 was chosen as the nuclear region because universal primers are available (White *et al*. 1990) and it is a multicopy gene. Due to the small body size of ostracods and their calcareous carapace, DNA extraction is difficult and single-copy genes are currently very hard to amplify.

We show that substitution rates in darwinulids are low, suggesting slow molecular evolution. Furthermore, intraspecific genetic variability differs significantly between mitochondrial and nuclear DNA, with zero substitutions in the ITS1 region of *Darwinula stevensoni*. Possible explanations for this surprising result are discussed.

2. MATERIAL AND METHODS

(a) *Populations*

Ostracods were sampled from lakes, ponds and rivers and stored in ethanol until DNA was extracted. *Darwinula stevensoni* was collected from 19 European sites (table 1) and from Lake Sibaya (South Africa).

Nuclear DNA from one to seven individuals from each site was analysed, resulting in a sample size of 56 individuals for ITS1. Mitochondrial DNA was sequenced for 17 individuals from 15 different sites, including the African one.

Microdarwinula zimmeri and *Darwinula brasiliensis* (both from Clue de la Fou, France) were collected as outgroups. Their taxonomic position within the Darwinulidae and in relation to *Darwinula stevensoni* will be discussed below.

(b) *Molecular analysis*

DNA was extracted by a modification of the chelex method (Walsh *et al*. 1991): ground tissue was incubated with a 5% chelex solution for 10 min at 100 °C and part of the supernatant was directly used for PCR without spinning it down. DNA was extracted from each individual separately. 'No-DNA' extractions were also conducted and carried through all stages of processing to check for contamination.

For both PCR and automatic sequencing, universal primers (White *et al*. (1990) for ITS1 and Folmer *et al*. (1994) for COI) were used first and the sequences obtained were checked. As no ITS sequences are available from other crustaceans, a BLAST search of GenBank was conducted which detected similarities with 18S and 5.8S rDNA from a variety of taxa and ITS1 from ticks, confirming the identity of the PCR product. Alignments with COI sequences from crustaceans also confirmed the identity of the mitochondrial sequences (e.g. 75.8% similarity with COI of *Penaeus vannanei* GenBank accession number X82503).

Species-specific primers were designed. PCRs were performed with the following temperature profiles and conditions in a TrioThermoblock (Biometra):

ITS1: 5 min 95 °C; 35 cycles with 1 min 95 °C, 1 min 50 °C, 2 min 72 °C; 10 min 72 °C, in a total volume of 25 µl with 200 µM dNTPs, 2.5 µl 10x PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl), 1.5 µM $MgCl_2$, 10 pmol primer ITS1D (5′-GGAAGGATCAC TGAGGTGT-3′), 10 pmol ITSY (5′-GTTCAAACTGTGGCGGTTTT-3′) and 0.5 U Thermoprime (Applied Biosystems).

Cytochrome oxidase I (COI): 5 min 95 °C; 35 cycles with 1 min 95 °C, 1 min 40 °C, 2 min 72 °C; 10 min 72 °C, in a total volume of 25 µl with 200 µM dNTPs, 2.5 µl 10x PCR buffer (10 mM Tris/HCl, pH 8.3, 50 mM KCl), 1.5 µM $MgCl_2$, 10 pmol primer HCOD (5′-ACTTCAGGGTGA CCAAAAAA-3′), 10 pmol LCOD (5′-CATAAAGATATTGGAACAAT-3′) and 0.5 U Thermoprime (Applied Biosystems).

Table 1. *Genetic variation in Darwinulidae*

(Genetic variation was estimated as mean gamma distance using a Kimura 2-parameter model (in substitutions per nucleotide) for each comparison with MEGA. pos = codon position; Dst = *Darwinula stevensoni*; Dbras = *Darwinula brasiliensis*; Micro = *Microdarwinula zimmeri*; Afr = Africa; Eur = Europe. The Darwinulidae are presently being revised and the 'Africana' lineage, to which *D. brasiliensis* belongs, will be described as a separate genus (Martens & Rossetti, in preparation). *Darwinula stevensoni* was collected from the following 20 sites: Pääjärvi (Finland), Semerwater (Great Britain), Blikkreek and Hollandersgatkreek (Belgium), Wörthersee, Keutschachersee, Mondsee, Ossiachersee, Faaker See, Afritzer See (all Austria), Lago di Endine, Lago di Monate, Lago di Garda, Comabbio, Montofarno, Mantova, Annone (all Italy), Clue de la Fou (France), Lake Bled (Slovenia), Lake Sibaya (South Africa). We sequenced ITS1 for one specimen of *Microdarwinula zimmeri* and three specimens of *D. brasiliensis* and COI for one specimen of each species.)

comparison	ITS1 all	COI all	COI pos1	COI pos2	COI pos3
Dst-Dbras	0.15	0.22	0.125	0.019	0.84
Dst-Micro	0.21	0.277	0.115	0.016	1.13
Dst-Afr/Eur	0.00	0.038	0.02	0.002	0.095
Dst Eur, within group I	0.00	0.004	0.005	0.00	0.006
Dst Eur, within group II	0.00	0.006	0.005	0.004	0.008
Dst Eur, among groups	0.00	0.022	0.012	0.003	0.051

PCR products were cleaned with the Wizard DNA purification kit (Pharmacia), and approximately 5 ng of the PCR product and 3.3 pmol of primer were used for cycle sequencing. Sequencing reactions were carried out in both directions, according to the manufacturer's protocol (25 cycles with 30 s 96 °C, 15 s 50 °C and 4 min 60 °C) with the PRISM Dye Terminator Cycle Sequencing Reaction kit (Applied Biosystems). Identical primers were used for both PCR and sequencing. In the case of the mitochondrial fragment, an additional set of internal primers was designed for sequencing (internal primers HCODII 5′-AATGTTTGCTGAAAGG GGG-3′ and LCODII 5′-CCCCCTTTCAGCAAACATT-3′). After cycle sequencing, extension products were cleaned with a phenol-chloroform extraction, and analysed with the ABI 373 machine.

To check for intra-individual variation, a cloning experiment was conducted. The ITS1 PCR product of one *D. stevensoni* individual was cleaned, ligated into pGEM Vector (Promega), following the manufacturer's protocol and then transformed into *E. coli* XL1 blue. Positive colonies were picked, amplified and minipreps made with the Wizard DNA extraction kit (Promega). Single clones were cycle-sequenced with the PCR primers.

(c) *Statistical analysis*

To verify individual sequences, the PCR product from each individual was sequenced in both directions, the two strands were aligned with PILEUP (using gap weights of 1, 5, or 10) in the Wisconsin GCG package and checked manually. Kimura two-parameter distances were estimated with MEGA (Kumar *et al*. 1993) and phylogenetic trees constructed with MacClade (Maddison & Maddison 1992) and PAUP (Swofford 1993) and bootstrapped with PHYLIP (Felsenstein 1993). Inserts and

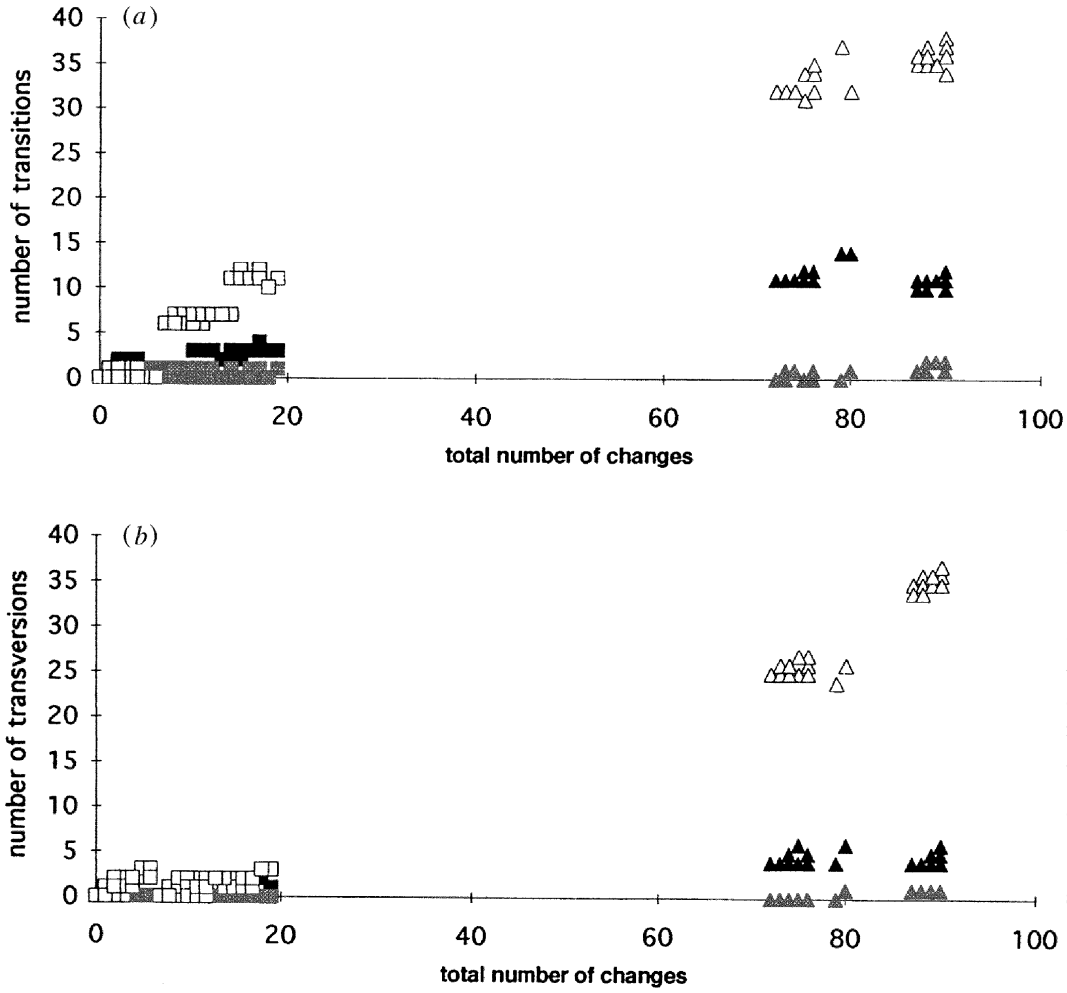

Figure 1. Number of transitions and transversions in COI of the darwinulids, compared to the total number of changes in COI. (*a*) Transitions. (*b*) Transversions. The three codon positions are indicated with black (1), grey (2) and white (3), taxonomic levels of comparison with square (intraspecific) and triangular (interspecific) symbols.

deletions were not included as valid characters for tree-construction but numbers of indels were noted. Only individuals sequenced for both ITS1 and COI were used for tree construction and statistical analysis. DNA sequences are available from GenBank with accession numbers AF031284 to AF031308.

3. RESULTS

(a) *Genetic variation*

Sequence divergence in ITS1 between *Darwinula stevensoni* and its two outgroups reached 15% (*D. brasiliensis*) and 21% (*Microdarwinula zimmeri*) (table 1), with five and four indels, respectively, at a gap weight of five. In the three individuals of *D. brasiliensis*, there were no substitutions but one individual differed from the other two by a 3 bp deletion. However, not a single substitution or indel was detected in the 320 bp of ITS1 within *D. stevensoni*—all 51 individuals from Europe and five from South Africa had an identical sequence.

All ten PCR products of ITS1 from one specimen of *Darwinula stevensoni* which were cloned and sequenced were 100% identical, indicating that this animal is homozygous and that there was little or no variation between tandem repeats of ITS1. Our ability to obtain clean sequences directly from amplification products for all specimens supports the conclusion that ITS1 sequence variation is absent within individuals.

In contrast to the data from nuclear DNA, the 447 bp of the mitochondrial COI gene showed both interspecific and intraspecific, genetic variation. Genetic distance to *D. brasiliensis* (22%) was, as for ITS1, slightly lower than to *M. zimmeri* (27.7%). African and European individuals of *D. stevensoni* differed with a maximum distance of 3.8%, European populations with a maximum of 2.2%. The number of substitutions in the three different codon positions of COI varies as expected: highest at third positions and lower for first and second positions (figure 1). There were no insertions or deletions.

(b) *Phylogenetic relationships*

A maximum parsimony tree, based on combined ITS1 and COI sequences, was constructed with MacClade (figure 2). *Darwinula brasiliensis* and *Microdarwinula zimmeri* were defined as outgroups. There is a clear separation between African and European populations of *D. stevensoni*. European populations of *D. stevensoni* fall into two groups (I and II), but no geographical pattern is observed: some Italian populations cluster with British and Belgian samples, others group with French and Austrian individuals. A tree constructed with neighbour joining showed the same topology down to the level of the two *D. stevensoni* clusters.

(c) *Fossil dating and rates of evolution*

Calibrating the observed genetic divergences with fossil records enables us to estimate evolutionary rates. From fossil analyses (describing the first appearance of a particular lineage in the fossils, thus reflecting minimal time scales), it appears that the origin of the '*africana*' lineage,

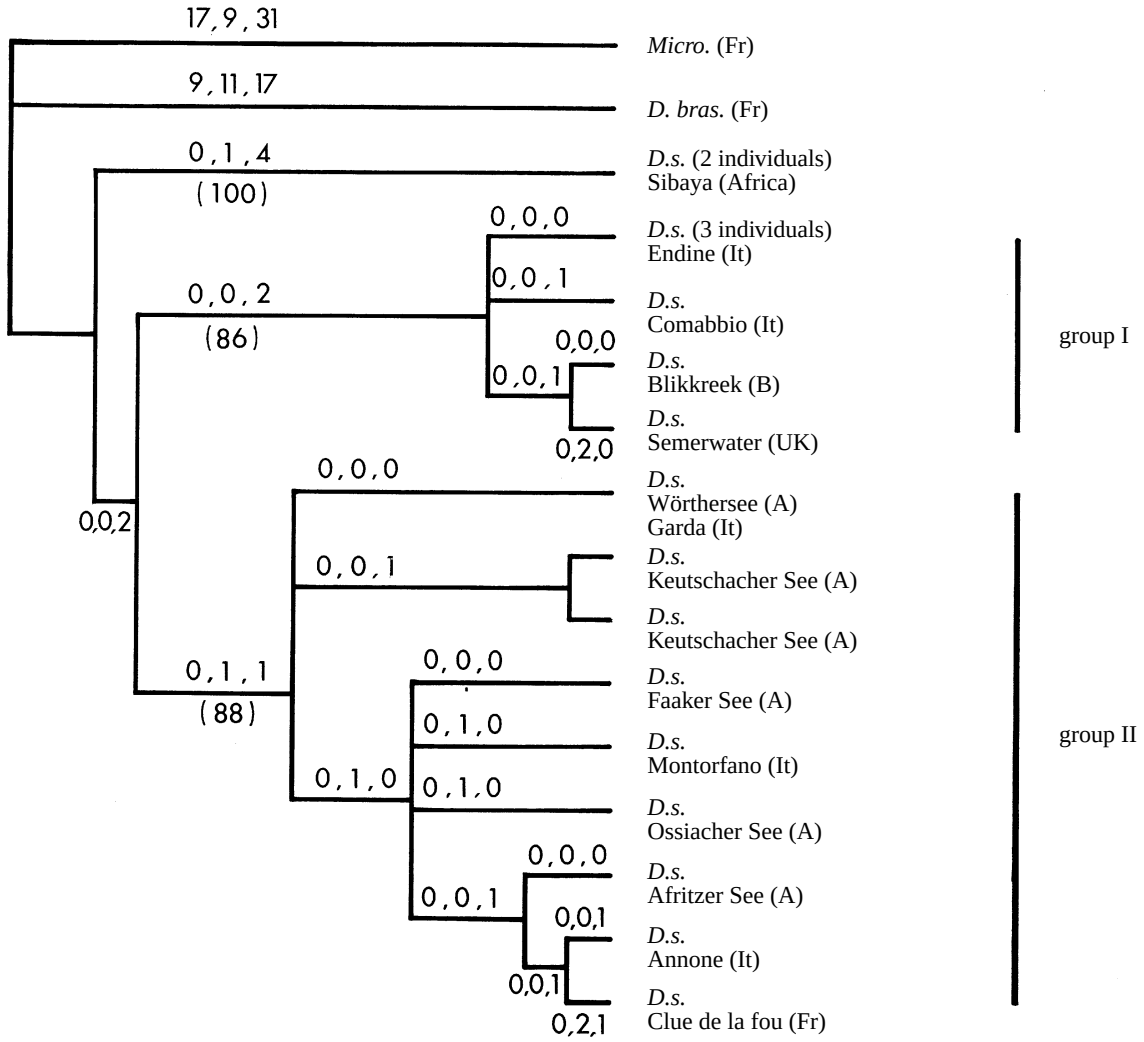

Figure 2. Cladogram, based on 447 bp of the COI gene and 320 bp of the ITS1 region for a set of congruent samples. The tree was constructed with MacClade (branch lengths are not proportional to distances) and represents one of several maximum parsimony trees that differ only in details of branching within clades I and II of *Darwinula stevensoni*. Insertions and deletions were needed to align ITS1 sequence data between the outgroups and *D. stevensoni*, but were excluded from the rate calculations and tree construction. Numbers given above the branches are the number of substitutions in ITS1, COI positions 1+2 and COI position 3, respectively. Bootstrap values (in percentage) are shown in parentheses for major branches.

to which *Darwinula brasiliensis* belongs (Martens *et al.* 1998), is situated at least 35 Ma (Huang & Wang 1988), probably 60 Ma (Zhang & Wu 1986) ago. Evolution of ITS1 (comparing *D. brasiliensis* and *D. stevensoni*) thus has a maximum subsitution rate of 0.13 to 0.22% per Ma and of COI a maximum rate of 0.19 to 0.32% per Ma (table 2). The first fossil record of *Microdarwinula* dates to 25 Ma (Straub 1952); molecular evolution is apparently faster in this lineage with 0.42% per Ma in ITS1 and 0.55% per Ma in COI (table 2). Note that the cited fossil records comprise minimal time scales: if older fossils were to be discovered, this would further reduce our estimates of substitution rate.

These estimated evolutionary rates can be used to calculate, from the observed intraspecific genetic variability of COI, that African and European populations of *Darwinula stevensoni* have diverged at least 4.4 Ma (maximum 7.6 Ma) ago (table 2). The split amongst European populations of *D. stevensoni* is estimated at 2.4 Ma (maximum 5.7 Ma) (table 2). Assuming that ITS1 within *D. stevensoni* evolves with a rate similar to the outgroups, a sequence divergence of 2.8% between African and European populations (equivalent to nine substitutions) and of 1.5% between European populations (being equivalent to five substitutions) would be expected (table 2), but no substitution was observed at all. This difference between expected and observed evolutionary rates of ITS1, relative to COI, within *D. stevensoni* is statistically significant (table 3). This conclusion does not depend on the absolute rate estimates, nor does it depend on the topology of phylogenetic relationships within *D. stevensoni*. Indels are also less frequent than expected within *D. stevensoni*. Changing the gap weight used to align the ITS1 sequences alters the estimations of numbers of indels and substitutions. A gap weight of 1.0 produced an alignment which implied 18 substitutions and seven indels among species. This minimal estimate does not alter the significance of the substitution rate reduction in *D. stevensoni*.

In principle, the significant result in table 3 could be caused by more rapid accumulation of COI substitutions in clonal lineages within *D. stevensoni*, relative to accumulation on branches to the other species. Comparison of the

Table 2. *Evolutionary rates in the Darwinulidae*

(Rates were estimated using gamma distances under the Kimura two-parameter model and fossil records. The time of evolution is double the time since two lineages split. Dst = *Darwinula stevensoni*; Dbras = *Darwinula brasiliensis*; Micro = *Microdarwinula*; Afr = Africa; Eur = Europe. (1) Observed divergence in per cent. (2) Mean substitution rate in percentage/Ma, calculated from the estimated rates for Dst/Dbras (maximal value) and Dst/Micro. (3) Time of evolution, calculated from (2) and the observed difference within Dst between African and European populations. (4) Expected divergence, calculated from (2) and (3).

comparison	locus	divergence (%)	time of evolution (Ma)	rate (%/Ma)
between species				
Dst-Dbras	COI	22.2 (1)	70[a]–120[b]	0.32–0.19
	ITS1	15.4 (1)	70[a]–120[b]	0.22–0.13
Dst-Micro	COI	27.7 (1)	50[c]	0.55
	ITS1	21.0 (1)	50[c]	0.42
within species				
Dst Afr/Eur	COI	3.8 (1)	8.8 (3)	0.44 (2)
	ITS1	0 (1)/2.8 (4)	8.8 (3)	0.32 (2)
Dst Eur	COI	2.1 (1)	4.8 (3)	0.44 (2)
	ITS1	0 (1)/1.5 (4)	4.8 (3)	0.32 (2)

[a]Huang & Wang (1988).
[b]Zhang & Wu (1986).
[c]Straub (1952).

Table 3. *Number of changes in the branches of the tree*

(Number of changes in the maximum parsimony tree shown in figure 1 for *D. stevensoni* were estimated for different codon positions and we then tested the null hypothesis of equal rates of ITS1 substitution within *D. stevensoni* and between *D. stevensoni* and the outgroups, relative to COI: ITS/COI (pos 1+2) $G_1 = 9.78$, $p < 0.01$; ITS/COI (pos 3) $G_1 = 7.82$, $p < 0.01$; ITS/COI (all pos) $G_1 = 10.02$, $p < 0.01$ (with Yates' correction in each case).)

	COI positions 1+2	COI position 3	ITS substitutions	ITS indels
intraspecific	10	15	0	0
interspecific	21	48	26	5

numbers of substitutions at different COI codon positions suggests that this is not due to saturation in the outgroup branches (figure 1), and this is further supported by the fact that the change of evolutionary rates within *D. stevensoni* remains significant when only first and second codon positions of COI are used (table 3). In addition, a likelihood analysis using PAML (Yang 1995) does not show a significant improvement in fit for a variable substitution rate model, as compared to a constant rate (molecular clock) model for any of the COI codon positions (2 × change in log-likelihood = 27.6, 16.0 and 30.4, d.f. = 19, for positions 1, 2 and 3, respectively). This means that the significant difference between nuclear and mitochondrial DNA in *D. stevensoni* is not caused by a speed-up in COI evolution, but by a slow-down in ITS1.

3. DISCUSSION

(a) *Low genetic variability*

Almost no data exist on sequence divergence of ITS1 and COI in ostracods. Schön *et al*. (1997) estimated maximal genetic distances of 10.3% (ITS1) and 20.9% (COI) between European populations of the freshwater ostracod *Eucypris virens*. Some of the samples investigated were asexual, but this species has a geographic pattern of parthenogenesis, with extant sexual populations around the Mediterranean. Although the histories of asexual reproduction are very different in *E. virens* and *D. stevensoni*, the results for *Eucypris* show that both loci can vary markedly within ostracod species. Studies on other taxa have reported low, but significant within-species variation in ITS1 and ITS2 for *Drosophila* (Schlötterer *et al*. 1994) and ticks (McLain *et al*. 1995). The complete absence of intraspecific variability in this part of the genome in *D. stevensoni* is, therefore, exceptional.

In contrast to the ITS1 results, we observed some intraspecific variation of *D. stevensoni* in COI. Comparing the maximum of 3.8% divergence with literature data on crustaceans (*Artemia;* Perez *et al*. 1994) and other invertebrates (Funk *et al*. 1994; Satta & Takahata 1990; Okamoto *et al*. 1995) shows that the intraspecific variability in mitochondrial DNA is not particularly low.

Calibrating the observed mitochondrial divergence between *D. stevensoni* and its outgroups with the fossil record (table 2) reveals that mitochondrial DNA in the Darwinulidae evolves at a maximum rate of 0.32–0.55% per Ma, which is much less than the evolutionary rate of COI in other crustaceans (2.2 to 2.6% per Ma; Knowlton *et al*. 1993) and only about half the minimal estimate of 0.8% for mitochondrial DNA in *Drosophila* (Sharp & Li 1989). On the same basis, the interspecific maximum substitution rate of 0.22–0.44% for ITS1 in the Darwinulidae is also approximately half that in *Drosophila*, where 1% per Ma has been estimated (Schlötterer *et al*. 1994). We should emphasize here that ostracods are invertebrates with an extensive and widely studied fossil record (Moore 1961) offering unique opportunities for reliable calibrations of the molecular clock.

Other studies on *D. stevensoni* show an absence of intraspecific morphological variation (Rossetti & Martens 1996), little allozyme variability (Havel & Hebert 1993; Rossi *et al*., in preparation) and only small morphological differences at the interspecific level in European Darwinulidae (Martens *et al*. 1998), possibly suggesting a general reduction in evolutionary rate.

(b) *Alternatives to slow substitution rate*

The observed zero variability of ITS1 within *D. stevensoni* could imply that the Recent species consists of one extremely successful clone, and the low morphological variation (Rossetti & Martens 1996) might support this. *Darwinula stevensoni* is unique among Darwinulidae as it is not only geographically widespread, but also common. All other species in Darwinuloidea are rare, occurring in 'marginal' habitats with little competition. It may be that a single clone of *D. stevensoni* has evolved a 'general purpose genotype' (Lynch 1984), which has allowed it to invade a

whole array of habitats: fresh water to saline, arctic to tropical conditions, rivers, lakes and interstitial bogs (Rossetti & Martens 1996). In a selective sweep, this 'super-clone' may have outcompeted all other clones and colonized at least Africa and Europe. If such a selective sweep occurred recently it could explain the sequence homogeneity in ITS1. In our opinion, there are several cogent arguments against this hypothesis.

In an apomictic lineage, a selective sweep will not only influence all nuclear loci, but also the mitochondrial genome. Therefore, the genetic variability we observe in the mitochondrial COI region in *D. stevensoni* is inconsistent with the recent selective sweep explanation, unless molecular evolution in COI accelerated considerably. Our observations do not support such a rate increase: evolutionary rates of COI in the Darwinulidae, calculated from reliable, fossil records (table 2) are slow compared with other crustaceans (Knowlton *et al*. 1993), there is no evidence for saturation in the outgroup branches, and likelihood analyses do not reveal significant rate variation. Note that in an apomictic lineage, there is no reason to expect a difference between accumulation of variation within a 'species' and divergence between species since all clones evolve independently.

A recent selective sweep would also require that colonization and competitive displacement occurred rapidly. However, we are not only dealing with an impressive geographical range (Finland to Southern Africa the latter in a very pristine locality where colonial influence was minimal and chances of recent introductions almost nil), but darwinulids are also poor colonizers relative to other non-marine ostracods. They are the only group which lacks the desiccation-resistant stages which are present in other taxa. In this respect, the time estimate for the split between the African and European lineages, based on COI divergence, is plausible. We cannot eliminate the possibility that an ancient selective sweep occurred where a successful clone displaced competitively inferior ones, but this must have happened millions of years ago. Eight to ten substitutions should have occurred since this event in the ITS1 region, if European and African lineages are compared. This has not happened, and this is significant.

A further alternative is that *Darwinula stevensoni* might, in fact, not be exclusively apomictic. The 'Meselson effect' of increasing divergence between alleles within one lineage, in the absence of recombination, was not observed in our ITS1 data. However, this may not argue against *D. stevensoni* being an ancient apomict. Tandemly repeated regions such as ITS1 show concerted evolution, due to unequal crossovers or gene conversions which tend to homogenize copies within sexual lineages (Birky 1996; Hillis & Dixon 1991) and these processes also operate in asexuals (Crease & Lynch 1991). Crease and Lynch (1991) found a lower genetic variability of rDNA in obligate parthenogenetic clones of *Daphnia* than in populations with cyclic sexual reproduction. A similar effect could account for our results but it is difficult to understand why such a process should only occur in *D. stevensoni* and not in the two outgroup species which also reproduce asexually.

Occasional sex could allow a nuclear variant to spread independently of the mitochondrial genome. The debate about males in *D. stevensoni* has continued for a century (see, for example, Little & Hebert 1996). Males were first reported and illustrated together with the original species description (Brady & Robertson 1870), as well as by Turner (1895) who mentioned a male of *D. improvisa* (a synonym of *D. stevensoni*). Unfortunately, the material appears to have been lost (Sohn 1987) and cannot be checked. Rossetti & Martens (1996) recently discussed the value of these old records: the specimen illustrated by Brady & Robertson (1870) could be genuine, the male described by Turner (1895) is most doubtful (see also Sohn 1987). Subsequently, neither screening of thousands of animals from different populations (McGregor 1969; Ranta 1979), nor culturing of *D. stevensoni* (Rossi, personal communication) has ever revealed males, unlike in *Limnocythere inopinata*, where similar efforts have repeatedly revealed rare male occurrences (Geiger, personal communication).

Even if rare males do occur in *D. stevensoni*, it is probable that they are atavistic and non-functional (Rossetti & Martens 1996) as in other asexual taxa such as *Artemia salina* (Browne 1992). Sexual reproduction would further require coincidental meiotic egg production by females: otherwise, fertilization of unreduced gametes leads to polyploidy. The frequency of polyploid clones in species with asexual reproduction could thus be a further indication of hybridization with males. This has been observed in other non-marine ostracods (Rossi & Menozzi 1994), but evidence of polyploidy was not found in allozyme analyses of more than 2000 individuals of *D. stevensoni* (Rossi *et al*. in preparation).

On the other hand, the chromosomal morphology of *D. stevensoni* fulfils the predictions for long-term apomicts (Judson & Normark 1996): chromosomes have an almost rounded shape and homologous pairs cannot be distinguished (Tetart 1978). To summarize, if they exist at all, rare males in *D. stevensoni* are in all probability non-functional.

Occasional automixis, as suggested for *Artemia* (Browne 1992) might also partially uncouple the divergence of nuclear and mitochondrial sequences by segregating nuclear genotypes within a mitochondrial DNA lineage. This could potentially break the complete association between nuclear and mitochondrial genomes expected under apomixis. However, no evidence for automixis has been found in ostracods and it is difficult to see how it would permit the spread of a nuclear genome without parallel homogenization of the mitochondrial genome.

Genetic variability of a neutral genomic region without recombination can be reduced by selection, assuming that selection acts against deleterious mutations elsewhere and the two regions are linked; this process is called background selection (Charlesworth 1994). Background selection can have considerable effects on the levels of intraspecific diversity (Charlesworth 1994) and might be another explanation for reduced genetic variability in self-fertilizing plants and genomic regions with restricted recombination (Charlesworth *et al*. 1993). Background selection cannot account for our observations for two reasons: in an apomictic lineage, the entire genome is linked, including the mitochondrial DNA, so that any effect on ITS1 should apply equally to COI; and background selection does not explain the significant difference between evolutionary rates within one asexual species (*D. stevensoni*), and rates among asexual species.

(c) *Low mutation rate and asexuality*

The arguments above suggest that for the moment, a reduced substitution rate in the ITS1 of clonal lineages of *Darwinula stevensoni* is the best explanation for the observed data. Indeed, the best estimate of the rate from our data is zero. Low substitution rates may be caused by many different processes (Mindell & Thacker 1996) but these fall into just two categories: effects on mutation rate and effects on fixation rate, primarily constraints imposed by selection but also the influence of concerted evolution in tandemly repeated loci. In an apomictic lineage selection operates on the entire genome. Although the sequence of the ITS region may be weakly constrained by selection in sexual species, it is extremely unlikely that this selection could dominate clonal substitution in an apomictic species. The effects of concerted evolution should apply equally to substitution rates in both inter- and intraspecific comparisons. Therefore, we consider reduced mutation rate to be the better explanation. We cannot say at present whether this reduction is specific to ITS1, or general across the nuclear genome. Sequencing more nuclear genes should strengthen our argument.

A low mutation rate may be advantageous for an apomictic lineage because it counters the effects of deleterious mutations (Hurst & Peck 1996). Note however that the reduction suggested here is not associated with the origin of asexuality but rather with a single successful species within an ancient asexual family. The widespread occurrence of *D. stevensoni*, with little clonal variation suggests that it has probably evolved a general purpose genotype. This strategy does not occur in other non-marine ostracod groups with asexual reproduction where populations consist of clusters of dozens of genotypes (Rossi & Menozzi 1994). It may, therefore, be advantageous for *D. stevensoni* to protect this successful genotype by reducing nuclear mutability.

The mutation rate is known to be influenced by the number of DNA replications per generation (Drake 1996). *Darwinula stevensoni* is a small ostracod species (0.7–0.8 mm), has a relatively long generation time of up to four years and a low reproductive rate (Ranta 1979). Its benthic habitat may be protected from some extrinsic mutagens like UV-B. *Darwinula stevensoni* carries its offspring in a brood pouch, perhaps allowing it to abort mutant embryos (Lively & Johnson 1994) and reducing the need for a large number of offspring. Depending on the female mutation rate, omitting sex itself can reduce the amount of genetic change (Redfield 1994). A polymerase with an improved fidelity or a lower metabolic rate might also be important (Mindell & Thacker 1996). The overall mutation rate may be reduced by any or all of these factors, but it is unlikely that they are sufficient to explain the observed, complete absence of ITS1 substitutions over several million years.

One other possibility is that DNA repair could further counter an already low mutation rate. Gabriel *et al*. (1993) suggested that asexuals could escape the accumulation of deleterious mutations by omitting repair. Moran (1996) reported the loss of one repair system in an asexual endosymbiont, supporting this idea (but see Schön *et al*. 1996). Alternatively, enhanced DNA repair, in the strict sense of correctly repairing a wrong base, may be advantageous in asexual lineages (Schön *et al*. 1996). We distinguish between the primary mutation rate which is the original rate of structural changes, upon which repair acts, and the resulting secondary mutation rate. The latter is the rate which influences the observed substitution rate, and which is exceptionally low in *D. stevensoni*. Repair mechanisms are independent in mitochondria (Avise 1994) which fits well with the observed difference between intraspecific mitochondrial and nuclear variability. As various repair mechanisms are known from eukaryotes (Friedberg *et al*. 1995), there is potential for the evolution of increased efficiency. Selection for mutability reduction should be much stronger without sex (Hurst & Peck 1996), thus highly efficient DNA repair may be particularly favoured in an ancient asexual.

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REFERENCES

Avise, J. C. 1994 *Molecular markers, natural history and evolution*. London: Chapman & Hall.

Birky, C. W. 1996 Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. *Genetics* **144**, 427–437.

Brady, G. S. & Robertson, D. 1870 The Ostracoda and Foraminifera of tidal rivers. *Ann. Mag. Nat. Hist.* **6**, 133, 307–309.

Browne, R. A. 1992 Population genetics and ecology of *Artemia*: insights into parthenogenetic reproduction. *Trends Ecol. Evol.* **7**, 232–237.

Butlin, R. K. & Griffiths, H. I. 1993 Ageing without sex? *Nature* **364**, 680.

Charlesworth, B. 1994 The effect of background selection against deleterious mutations on weakly selected, linked variants. *Genet. Res.* **63**, 213–227.

Charlesworth, B., Morgan, M. T. & Charlesworth, D. 1993 The effect of deleterious mutations on neutral molecular variation. *Genetics* **134**, 1289–1303.

Crease, T. J. & Lynch, M. 1991 Ribosomal DNA variation in *Daphnia pulex*. *Molec. Biol. Evol.* **8**, 620–640.

Drake, J. W. 1996 The antievolutionary component of antimutagenesis and anticarcinogenesis: where do mutation rates come from and where are they going? *Mut. Res.* **350**, 58.

Felsenstein, J. 1993 *PHYLIP—phylogeny inference package, version 3.5*. Seattle, WA: Department of Genetics, University of Washington.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. 1994 DNA primers for amplification of mitochondrial Cytochrome C oxidase subunit I from diverse metazoan invertebrates. *Molec. Mar. Biol. Biotechn.* **3**, 294–299.

Friedberg, E. C., Walker, G. C. & Siede, W. 1995 *DNA repair and mutagenesis*. Washington: ASM Press.

Funk, D. J., Futuyama, D. J., Orti, G. & Meyer, A. 1994 Mitochondrial DNA sequences and multiple data sets: a phylogenetic study of phytophagous beetles (Chrysomelidae: *Ophraella*). *Molec. Biol. Evol.* **12**, 627–640.

Gabriel, W, Lynch, M. & Burger, R. 1993 Muller's ratchet and mutational meltdowns. *Evolution* **47**, 1744–1757.

Griffiths, H. I. & Butlin, R. K. 1994 *Darwinula stevensoni*: a brief review of the biology of a persistent parthenogen. In *The*

evolutionary ecology of reproductive modes in non-marine Ostracoda (ed. D. J. Horne & K. Martens), pp. 43–55. Greenwich, UK: University Press.

Havel, J. E. & Hebert, P. D. N. 1989 Apomictic parthenogenesis and genotypic diversity in *Cypridopsis vidua* (Ostracoda: Cyprididae). *Heredity* **62**, 383–392.

Havel, J. E. & Hebert, P. D. N. 1993 Clonal diversity in parthenogenetic ostracodes. In *Ostracoda in the Earth and life sciences* (ed. K. G. McKenzie & P. J. Jones), pp. 353–368. Rotterdam: Balkema.

Hillis, D. M. & Dixon, M. T. 1991 Ribosomal DNA: molecular evolution and phylogenetic inference. *Quart. Rev. Biol.* **66**, 411453.

Huang, B.-R. & Wang, S.-Q. 1988 Eocene ostracods from Shanxi and Shandong provinces. *Acta Micropalaeontologica Sinica* **5**, 385–394, pls III.

Hurst, L. D. & Peck, J. R. 1996 Recent advances in understanding of the evolution and maintenance of sex. *Trends Ecol. Evol.* **11**, 46–52.

Judson, O. P. & Normark, B. B. 1996 Ancient asexual scandals. *Trends Ecol. Evol.* **11**, 41–46.

Knowlton N., Weight, L. A., Solorzano, L. A., Mills, D. E. K. & Bermingham, E. 1993 Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. *Science* **260**, 1629–1632.

Kondrashov, A. S. 1993 Classification of hypotheses on the advantage of amphimixis. *J. Hered.* **84**, 372–387.

Kumar, S., Tamura, K. & Nei, M. 1993 *Molecular evolutionary genetics analysis* (MEGA *Version 1.01*). Pennsylvania State University.

Little, T. J. & Hebert, P. D. N. 1996 Ancient asexuals: scandal or artifact? *Trends Ecol. Evol.* **11**, 296.

Lively, C. M. & Johnson, S. G. 1994 Brooding and the evolution of parthenogenesis: strategy models and evidence from aquatic invertebrates. *Proc. R. Soc. Lond.* B **256**, 89–95.

Lynch, M. 1984 Destabilizing hybridization, general-purpose genotypes and geographic parthenogenesis. *Quart. Rev. Biol.* **59**, 257–290.

Maddison, W. P. & Maddison, D. R. 1992 *MacClade*, *version 3.0.* Sunderland, MA: Sinauer Associates.

Martens, K., Rossetti, G. P. & Fuhrmann, R. 1998 Pleistocene and recent species of the family Darwinulidae Brady & Norman, 1889 (Crustacea, Ostracoda) in Europe. *Hydrobiologia*. (In the press.)

Maynard Smith, J. 1986 Contemplating life without sex. *Nature* **324**, 300–301.

Maynard Smith, J. 1989 *Evolutionary genetics.* Oxford University Press.

McGregor, D. L. 1969 The reproduction potential, life history and parasitism of the freshwater ostracod *Darwinula stevensoni* (Brady and Robertson). In *The taxonomy, morphology and ecology of recent Ostracoda*. (ed. J. W. Neale), pp. 194–221. Edinburgh, UK: Oliver & Boyd.

McLain, D. K., Wesson, D. M., Collins, F. H. & Oliver, J. H. 1995 Evolution of the rDNA spacer, ITS2, in the ticks *Ixodes scapularis* and *I. pacificus* (Acari: Ixodidae). *Heredity* **75**, 303–319.

Mindell, D. P. & Thacker, C. E. 1996 Rates of molecular evolution: phylogenetic issues and applications. *A. Rev. Ecol. Syst.* **27**, 279–303.

Moore, R. C. 1961 *Ostracoda. Treatise on invertebrate palaeontology, part 9.* University of Kansas Press.

Moran, N. A. 1996 Accelerated evolution and Muller's ratchet in endosymbiotic bacteria. *Proc. Natn. Acad. Sci. USA* **93**, 2873–2878.

Muller, H. J. 1964 The relation of recombination to mutational advance. *Mut. Res.* **1**, 29.

Okamoto, M., Bessho, Y., Kamiya, M., Kurosawa, T. & Horii, T. 1995 Phylogenetic relationships within *Taenia taeniaformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the cytochrome *c* oxidase subunit I gene. *Parasitol. Res.* **81**, 451–458.

Perez, M. L., Valverde, J. R., Batuecas, B., Amat, F., Marco, R. & Garesse, R. 1994 Speciation in the *Artemia* genus: mitochondrial DNA analysis of bisexual and parthenogenetic brine shrimps. *J. Molec. Evol.* **38**, 156–168.

Ranta, E. 1979 Population biology of *Darwinula stevensoni* (Crustacea, Ostracoda) in an oligotrophic lake. *Ann. Zool. Fenn.* **16**, 28–35.

Redfield, R. J. 1994 Male mutation rates and the cost of sex for females. *Nature* **369**, 145–147.

Rossetti, G. P. & Martens, K. 1996 Redescription and morphological variability of *Darwinula stevensoni* (Brady & Robertson, 1870) (Crustacea, Ostracoda). *Bull. Konink. Belg. Inst. Natuurwetenschappen—Biologie* **66**, 73–92.

Rossi, V. & Menozzi, P. 1994 Enzyme and DNA polymorphism in ostracod evolutionary ecology. In *The Evolutionary ecology of reproductive modes in non-marine Ostracoda* (ed. D. J. Horne & K. Martens), pp. 43–55. Greenwich, UK: University Press.

Satta, Y. & Takahata, N. 1990 Evolution of *Drosophila* mitochondrial DNA and the history of the *melanogaster* subgroup. *Proc. Natn. Acad. Sci. USA* **87**, 9558–9562.

Schlötterer, C., Hauser, M.-T., Von Haeseler, A. & Tautz, D. 1994 Comparative evolutionary analysis of rDNA ITS regions in *Drosophila*. *Molec. Biol. Evol.* **11**, 513–522.

Schön, I., Martens, K. & Rossi, V. 1996 Ancient asexuals: scandal or artifact? *Trends Ecol. Evol.* **11**, 296–297.

Schön, I., Gandolfi, A., Di Masso, E., Rossi, V., Griffiths, H. I. & Butlin, R. K. 1997 Exceptionally high genetic variability among sexual and asexual lineages of *Eucypris virens* (Crustacea, Ostracoda). (Submitted.)

Sharp, P. M. & Li, W. -H. 1989 On the rate of DNA sequence evolution in *Drosophila*. *J. Molec. Evol.* **28**, 398–402.

Sohn, I. G. 1987 The ubiquitous ostracode *Darwinula stevensoni* (Brady & Robertson, 1870), redescription of the species and lectotype designation. *Micropalaeontology* **33**, 150–163.

Straub, E. W. 1952 Mikropalaeontologische Untersuchungen im Tertiär zwischen Ehingen und Ulm a. d. Donau. *Geol. Jb.* **66**, 433–523.

Swofford, D. L. 1993 *PAUP. Phylogenetic analysis using parsimony, version 3.1.1.* Champaign, IL: Illinois Natural History Survey.

Tetart, J. 1978 Les garnitures chromosomique des Ostracodes d'eau douce. *Travaux Lab. Hydrobiol. Pisiculture Univers. Grenoble* **69–70**, 113–140.

Turner, C. H. 1895 Freshwater Ostracoda of the United States. *Minnesota Geol. Nat. Hist. Surv. Zool.* **2**, 277–337.

Walsh, P. S., Metzger, D. A. & Higuchi, R. 1991 Chelex® 100 as medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* **10**, 506–513.

White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990 Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR protocols: a guide to methods and applications*, pp. 315–322.

Yang, Z. 1995 *PAML, phylogenetic analysis by maximum likelihood, version 1.1.* Pennsylvania State University.

Zhang, S. & Wu, Q.-Y. 1986 Cretaceous–Early Tertiary ostracods from the Lingbao Basin of Western Henan Province. *Acta Micropalaeontologica Sinica* **3**, 409–429, pls I–III.