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No slave to sex

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Fully asexual lineages cannot purge accumulating mutations from their genome through recombination. In ancient asexuals that have persisted without sex for millions of years, this should lead to high allelic divergences (the 'Meselson effect') as has been shown for bdelloid rotifers. Homogenizing mechanisms can counter this effect, resulting in low genetic diversity within and between individuals. Here, we show that the ancient asexual ostracod species *Darwinula stevensoni* has very low nucleotide sequence divergence in three nuclear regions. Differences in genetic diversity between embryos and adults furthermore indicate that up to half of the observed genetic changes in adults can be caused by somatic mutations. Likelihood permutation tests confirm the presence of gene conversion in the multi-copy internal transcribed spacer sequence, but reject rare or cryptic forms of sex as a general explanation for the low genetic diversity in *D. stevensoni*. Other special mechanisms (such as highly efficient DNA repair) might have been selected for in this ancient asexual to overcome the mutational load and Muller's ratchet. In this case, our data support these hypotheses on the prevalence of sex, even if the two extant ancient asexual groups (bdelloids and darwinulids) seem to follow opposite evolutionary strategies.

Keywords: ancient asexuals; Meselson effect; mutational load; gene conversion; DNA repair; asexual reproduction

1. INTRODUCTION

Several hypotheses predict that asexual lineages are doomed to early extinction (Maynard Smith 1978, 1998; Butlin 2002). Nevertheless, a few groups have apparently survived without sex for many millions of years. These ancient asexual scandals (Judson & Normark 1996), so-called because they defy ruling evolutionary theory, are assumed to carry genetic evidence of their long-term asexuality. The latter has been demonstrated for the bdelloid rotifers (asexual for *ca*. 30 Myr), which show high allelic divergence at the DNA sequence level (Mark Welch & Meselson 2000). This is known as the 'Meselson effect': through non-homologous accumulation of mutations, allelic divergences in individuals should be larger in long-term asexuals than in sexuals. Although this effect is accepted as a genetic test for the status of ancient asexuality, its powers are asymmetrical. Presence of the effect indicates long-term amixis but its absence does not necessarily imply sex (Butlin 2000). Low allelic divergences can also be caused by homogenizing mechanisms such as gene conversion (Butlin 2000) between alleles or highly efficient DNA repair (Schön & Martens 1998). Comparative research on ancient asexuals constitutes an alternative approach to test some of the more than 20 hypotheses related to the 'queen of problems in evolutionary biology' (i.e. the prevalence of sex in the living world; Bell 1982), especially those referring to the irreversible accumulation of (deleterious) mutations (Kondrashov 1988) and to the stochastic loss of least-corrupted genotypes (Muller's ratchet; Muller 1964). If special mechanisms are developed to counter the deleterious effects in the absence of sex, the validity of the hypotheses is supported.

Here, we test for the Meselson effect in a representative of the ostracod family Darwinulidae, the second putative ancient asexual animal group beside bdelloid rotifers. The fossil record shows that the ostracod family Darwinulidae has reproduced fully asexually for 200 Myr (Martens *et al.* 2003) and neither sexual nor mixed extant populations or close sexual relatives are known (Martens 1998). The type species *Darwinula stevensoni* has been fully asexual for 20–25 Myr (Straub 1952), which makes it the longest living, extant asexual species known. It is common, cosmopolitan and ubiquitous (Rossetti & Martens 1996, 1998), with a general purpose genotype (Van Doninck *et al.* 2002) and has, for an animal less than 1 mm in size, an unusually long lifespan of up to 4 years in subarctic populations (Ranta 1979).

Morphological (Rossetti & Martens 1996, 1998) and allozyme (Rossi *et al.* 1998) studies show a low variability in *D. stevensoni*. Estimates of genetic variability at the DNA level have so far been published from the nuclear internal transcribed spacer (ITS) 1 region and the mitochondrial cytochrome oxidase subunit I (COI) gene, showing no (Schön *et al.* 1998) or little (Gandolfi *et al.* 2002) divergence in ITS1. Neither of these studies constitute a test for the Meselson effect, either because direct sequencing was used (Schön *et al.* 1998) or because the sample scheme was too restricted (Gandolfi *et al.* 2002) to draw conclusions about allelic divergence of a multiple copy region.

We have estimated nucleotide sequence divergence of *D. stevensoni* within and between individuals from natural populations over a wide geographical range including South Africa, southern France, Italy, Belgium and Ireland. Three nuclear regions have been PCR-amplified, cloned and sequenced from individual ostracods: intron and exon regions of the single copy gene *hsp82*, the interspersed regions ITS1/ITS2 and a calmodulin intron (CadDs). Comparisons are made with sequence data of *hsp82* from *Cyprideis torosa*, a fully sexual ostracod species.

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Table 1. Mean genetic distance of *Cyprideis torosa* and *Darwinula stevensoni*, estimated as HKY 85 (Hasegawa *et al.* 1985) within and between populations.
(CI, confidence interval; B, Belgium; F, France; EU, between different European populations; EU–AFR, between European and South African populations; I, between two Italian populations. Belgian individuals are either adult females (A) or embryos (E). All other individuals are adult females. 95% CI in the *C. torosa* dataset is especially high owing to the presence of a single, very different clone in individual Ct1; the variable positions of this clone are mainly located in the intron region of *hsp82*.)

genomic region	species	comparison	number of comparisons	mean HKY 85	95% CI
hsp82	*C. torosa*	B, A	13	0.0497	0.0294
hsp82	*D. stevensoni*	B, A	24	0.0145	0.0046
hsp82	*D. stevensoni*	B, E	14	0.0056	0.0007
hsp82	*D. stevensoni*	F	26	0.0073	0.0004
hsp82	*D. stevensoni*	EU	66	0.0110	0.0002
hsp82	*D. stevensoni*	EU–AFR	71	0.0110	0.0004
ITS	*D. stevensoni*	I	12	0.0019	0.0006
CadDs	*D. stevensoni*	EU	16	0.0034	0.0006
CadDs	*D. stevensoni*	EU–AFR	24	0.0051	0.0004

2. MATERIAL AND METHODS

(a) *Ostracods*

Specimens of the ancient asexual ostracod species *D. stevensoni* were collected from the following lakes, rivers and springs: Hollandersgatkreek (Belgium), Lake Sibaya (Republic of South Africa), Lough Ballyquircke (Ireland), 'Cava' and 'Canale' of Mantova, (Italy) and Clue de la Fou (France). Adult females of the sexual ostracod species *C. torosa* were sampled from Hollandersgatkreek (Belgium). All ostracods were stored in 95% ethanol or frozen at −20 °C before DNA extraction.

(b) *PCR amplification, cloning and sequencing*

DNA was extracted from individual ostracods with a modified Chelex method (Schön *et al.* 1998); individual embryos were manually removed from the brooding pouch of living, adult females before DNA extraction. Eight hundred and fifty base pairs of *hsp82* containing intron and exon regions were amplified with primers hsp8.X (5′-ACGTTCTAGARTGRTCYTCCCA-RTCRTTNGT) and hsp1.2 (5′-TGCTCTAGAGCACARTT-YGGTGTNGGTTTYTA) and the following PCR protocol in a Progene DNA amplificator (Techne): 5 min at 95 °C, 10 × 50 s at 95 °C, 50 s at 50 °C, 2 min at 72 °C, 35 × 50 s at 95 °C, 50 s at 55 °C, 2 min at 72 °C; 10 min at 72 °C. Amplification occurred in 25 µl volumes with 0.5 U Taq DNA Polymerase (Amersham Pharmacia Biotech) and the provided buffer, 1.5 mM $MgCl_2$, 200 µmM dNTPs (Promega), 10 pmol of each primer and *ca.* 10 pg DNA. The 890 base pair (bp) stretch of ITS was amplified (with primers ITS1 and ITS4) as given (White *et al.* 1990; Schön *et al.* 1998) and 411 bp of CadDs as in Schön (2001). Cleaning and cloning of PCR products as well as DNA preparation from positive clones and sequencing occurred as previously described (Schön 2001).

(c) *Sequence analyses*

Processed sequences were viewed with Chromas 1.45, aligned with Clustal W (Thompson *et al.* 1994) and checked manually. Variable sites were identified by eye and by using the Sequence computer package (B. Schüren). Genetic and taxonomic identity of all sequences was confirmed by Blast (Altschul *et al.* 1997). A total of 129 cloned sequences were analysed in both directions.

Genetic distances were estimated with Paup v. 4.05 (Swofford 1998), using the HKY85 (Hasegawa *et al.* 1985) method under the assumption of equal rates for variable sites. For estimates at different levels (individuals, within and between populations), values of the lower levels were excluded. For example, for comparisons within populations, no intra-individual estimates were used. Cloning with subsequent sequencing can generate technical artefacts owing to Taq errors or mutations in the bacterial host. Obvious errors (point mutations and indels restricted to single clones) were excluded before further analysis; original and edited data matrices can be obtained from the first author on request. A subset of the edited sequences has been submitted to GenBank, submission numbers AJ534952–AJ534968.

(d) *Statistical analyses*

Student's *t*-tests and tests for correlations were conducted with the program Statistica. If different populations were tested against each other, geographically closest populations were chosen (e.g. *hsp82* from the South of France was tested against ITS from Italy). Details of the statistical analyses can be obtained from the first author on request. Permutation likelihood tests (McVean *et al.* 2002) were conducted with 'no frequency cut-off of rare alleles', Watterson theta estimate (θ_W) of population mutation rates, $\rho = 20$ and 10 000 permutations. Owing to the low number of segregating sites (0–6), tests were not applicable to individual *hsp82* sequences of *D. stevensoni* from South Africa and individual ITS and CadDs sequences. Highest composite log likelihoods were obtained with $4N_er = 10$ and 10 grid points for *D. stevensoni* (*D.s.*) and $4N_er = 200$ and 100 grid points for *C. torosa*. Values of p had similar significance levels for all datasets but ITS, if $4N_er = 20$ or 50 (*D.s.*) were used. $p_{|r2,\,d|}$ and $p_{|D',\,d|}$ are calculated by methods similar to published permutation-based tests (McVean *et al.* 2002) and describe the correlation between recombination and physical distance. $p_{\mathrm{Lk}_{max}}$ and $p_{\mathrm{Lk}_{max}}$ for gene conversion (GC) are new parameters of likelihood permutation tests (McVean *et al.* 2002).

3. RESULTS

We found that genetic divergence of *hsp82* is significantly higher for the sexual species *C. torosa* than for the ancient asexual *D. stevensoni*, both within individuals (mean HKY 85 (Hasegawa *et al.* 1985) distances of 0.090 and 0.0089, respectively; not shown in table 1) and between individuals from the same population (table 1; *t*-test: $p < 0.001$). In *D. stevensoni*, mean genetic diversity of

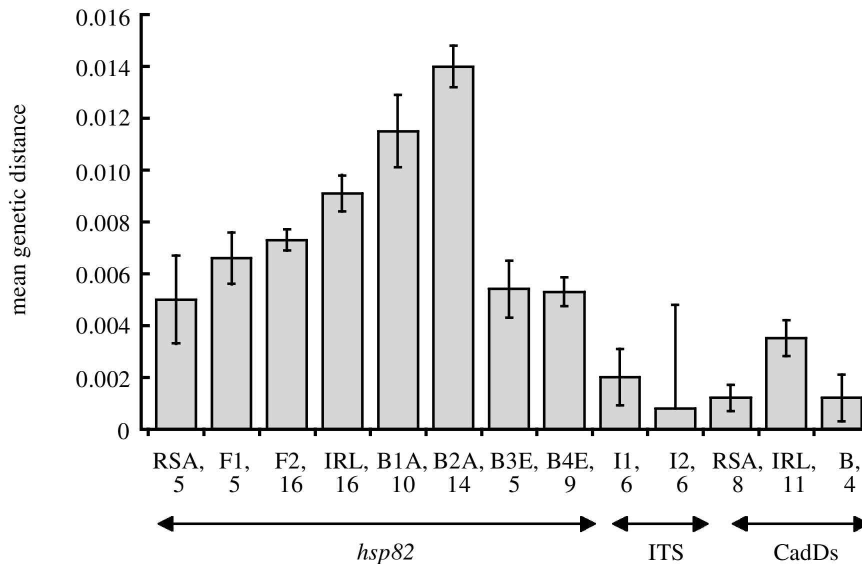

Figure 1. Mean genetic distances (HKY 85; Hasegawa *et al.* 1985) of *hsp82*, ITS and CadDs within individual *Darwinula stevensoni* genomes. Error bars indicate 95% CIs of means. Numbers below individual codes indicate numbers of sequenced clones from each PCR product. Unless specifically mentioned, all individuals are adult females. *hsp82*: RSA, Republic of South Africa; F1,2, two specimens from France; IRL, Ireland; B1A, B2A, two specimens from Belgium; B3E, B4E, two embryos from Belgium; I1, 2, two specimens from Italy; B, Belgium.

hsp82 is low at all levels of comparison: within individuals (figure 1), within and between populations (table 1). Mean genetic divergence of both ITS and CadDs in *D. stevensoni* (maximum of 0.002 and 0.0036, respectively) is significantly smaller than that of *hsp82* (table 1; figure 1; *t*-test: $p < 0.0001$) at the individual and population level.

Also, Watterson theta estimates (see table 2; 0.002 28–0.006 14) indicate low mutation rates of *D. stevensoni* in all examined genomic regions or low effective population size.

There is a strong effect of age class on genetic variability of *hsp82*: means in embryos reach only half of those in adults (table 1; figure 1; *t*-test: $p < 0.0001$) at the individual and at the population levels. Genetic divergences of *hsp82* from the same geographical region are of the same order of magnitude for individuals and populations (*t*-test: $p > 0.37$). Throughout Europe, however, we observe an effect of latitude on genetic variability for *hsp82*: both individuals and populations show significantly higher, mean genetic divergence of *hsp82* at northern latitudes than in southern locations (*t*-test: $p < 0.0001$). The pattern is not as clear for the genetic variability of the CadDs region for which fewer individuals were analysed.

New and more powerful likelihood permutation tests (McVean *et al.* 2002) based on the Fisher–Wright population model determine whether recombination or mutations have generated the observed pattern of nucleotide sequence divergence (which is small but present) in *D. stevensoni*. Multiple mutations are herewith regarded as the null hypothesis, whereas significant deviations of the estimated parameters indicate recombination. Sequence data of the sexual ostracod species *C. torosa* were also analysed with likelihood permutation tests to verify the validity of the tests.

As expected, highly significant *p* values were obtained for *hsp82* in the population of this sexual ostracod for all three permutation parameters (see table 2). The outcome is similar, if the single, highly variable sequence of one of the two individuals is excluded (not shown). In *hsp82* of *D. stevensoni*, only one significant value ($p_{|r2,\ d|}$) is found when sequences of all screened populations are pooled (table 2). No evidence for recombination appears if sequence data from each population of *D. stevensoni* are analysed separately.

We have also tested for cryptic (intra-individual) forms of sex by repeating the analyses with individual *hsp82* datasets from *D. stevensoni* (table 3). In this case, the test only picks up a single signature of $p_{|r2,\ d|}$ in one specimen from the Belgian population for which the maximal genetic divergence of *hsp82* was observed. The result is not confirmed by $p_{\mathrm{Lk}_{\max}}$, which is considered to be the most powerful parameter (McVean *et al.* 2002). Permutation likelihood tests indicate some recombination in the ITS region (significant $p_{\mathrm{Lk}_{\max}}$; see table 2); this result is congruent with the signal for gene conversion. No recombination is found in the CadDs region (table 2).

Likelihood permutation tests can indeed be modified to test for gene conversion instead of recombination with genetic change (G. McVean, personal communication). If the modified tests are used, significant deviations are only found in *hsp82* from *C. torosa* and ITS from *D. stevensoni* (tables 2 and 3).

4. DISCUSSION

Compared with the sexual ostracod species *C. torosa*, *D. stevensoni* shows significantly lower levels of genetic diversity within and between individuals. Mean genetic diversity in *D. stevensoni* is very low for all three examined genomic regions and for all levels of comparison: within and between individuals and populations. The low estimates for comparisons between populations are especially puzzling. Such low genetic divergences are opposite to the

Table 2. Results of the likelihood permutation test (McVean *et al.* 2002) for recombination or gene conversion (GC) (last column) at population or higher levels.
(Tajima D values were calculated for segregating sites including all alleles. Negative D indicates an excess of rare variants (McVean *et al.* 2002). θ_W, Watterson theta estimate of population mutation rate; $|r2, d|$ and $|D', d|$ are calculated by methods similar to published permutation-based tests (McVean *et al.* 2002), Lk_{max} and Lk_{max} GC are new parameters of likelihood permutation tests (McVean *et al.* 2002). All parameters describe the correlation between recombination and physical distance. *C.t.*, *Cyprideis torosa*; *D.s.*, *Darwinula stevensoni*; B, Belgium; A, adult; E, embryo; F, France; I, Italy. Bold p values are significant at the 0.01 level.)

dataset	species	number of individuals	number of clones	number of segregating sites	θ_W	D	$p_{Lk_{max}}$	$p_{\|r2, d\|}$	$p_{\|D', d\|}$	$p_{Lk_{max}}$ GC
hsp82, B	*C.t.*	2	13	130	0.104 97	−1.395	0.006	**0.0**	**0.0**	**0.0**
hsp82, all data	*D.s.*	8	80	25	0.006 01	0.365	0.7820	**0.0051**	0.1025	0.5110
hsp82, B, A	*D.s.*	2	24	19	0.006 14	1.162	0.4980	0.0218	0.0872	0.1940
hsp82, B, E	*D.s.*	2	14	10	0.003 77	1.808	0.7240	0.5888	0.4391	0.3690
hsp82, F	*D.s.*	2	21	13	0.004 10	0.707	0.920	0.3758	0.7807	0.6990
ITS, I	*D.s.*	2	12	6	0.002 28	−0.905	**0.0**	0.2022	0.2022	**0.0**
CadDs, all data	*D.s.*	4	24	4	0.002 62	0.947	0.2370	0.3427	0.3427	0.0760

Table 3. Results of the likelihood permutation test (McVean *et al.* 2002) for recombination or GC (last column) in *hsp82* of individual *Darwinula stevensoni*.
(Tajima D values were calculated for segregating sites including all alleles. Negative D indicates excess of rare variants (McVean *et al.* 2002). θ_W, Watterson theta estimate of population mutation rate; $|r2, d|$ and $|D', d|$ are calculated by methods similar to published permutation-based tests (McVean *et al.* 2002), Lk_{max} and Lk_{max} GC are new parameters of likelihood permutation tests (McVean *et al.* 2002). All parameters describe the correlation between recombination and physical distance. B, Belgium; A, adult; E, embryo; IRL, Ireland; F, France. Bold p values are significant at the 0.01 level.)

individual	number of clones	number of segregating sites	θ_W	D	$p_{Lk_{max}}$	$p_{\|r2, d\|}$	$p_{\|D', d\|}$	$p_{Lk_{max}}$ GC
B1 A	10	11	0.004 67	1.510	0.4300	0.3103	0.3759	0.2570
B2 A	14	19	0.007 21	0.638	0.6670	**0.0029**	0.0224	0.2940
B3 E	5	10	0.004 42	0.933	0.0980	0.9865	0.9054	0.0550
B4 E	9	9	0.005 18	0.954	0.5020	0.6672	0.6672	0.0760
IRL	16	12	0.004 35	1.822	0.6710	0.1893	0.0903	0.2050
F1	5	9	0.003 82	1.096	0.6380	0.2910	0.2910	0.7650
F2	16	12	0.004 35	0.370	0.9820	0.2775	0.2734	0.9550

prediction of the Meselson effect (Mark Welch & Meselson 2000) and confirm the exceptionally low genetic variability in *D. stevensoni* at the population level obtained by direct sequencing (Schön *et al.* 1998). Low Watterson estimates cannot be explained by small population size, as the number of individual *D. stevensoni* in certain patches can reach millions (Ranta 1979; Van Doninck *et al.* 2003).

The *in vivo* genetic variability of this ancient asexual might even be less than the obtained estimates, as *Taq* errors were treated conservatively (see § 2). Owing to the overall low number of variable sites in *D. stevensoni* sequences, we are working at the edge of the accuracy of cloning and sequencing methods. This is reflected in the slightly higher genetic variability of *D. stevensoni* in preliminary cloning experiments of ITS1 from a limited geographical region (Gandolfi *et al.* 2002) which is most likely due to a combination of technical artefacts (e.g. PCR products exposed to ultraviolet).

Also, not all of the observed genetic diversity is necessarily heritable. To distinguish between genetic changes in germ line and soma, embryos of *D. stevensoni* from the Belgian population, for which the maximal, intra-individual, genetic diversity was observed, were also screened. On average, genetic changes in embryos were only half of those observed in adults. Adults contain more than two sequences of *hsp82* within the same individual, which differ only slightly from each other in variable positions. Also, no obvious difference in the genetic variability between the exon and intron regions of *hsp82* could be observed. All this shows that mutations in adults can have a somatic origin, and might even constitute up to 50% of the genetic variability. These results are also in accordance with the observed, geographical pattern on mean genetic divergence within adults. Individuals are expected to acquire more somatic mutations the longer they live. Given the relatively long lifespan of the small animal (see above), this is applicable here. We observed that latitude significantly affects genetic variability of *hsp82* within Europe. Both individuals and populations show significantly higher, mean genetic divergence of *hsp82* at northern latitudes (where animals live up to 4 years; Ranta 1979) than in southern locations (with a shorter lifespan; Gandolfi *et al.* 2001; Van Doninck *et al.* 2003). A similar correlation between age and level of somatic mutations has been

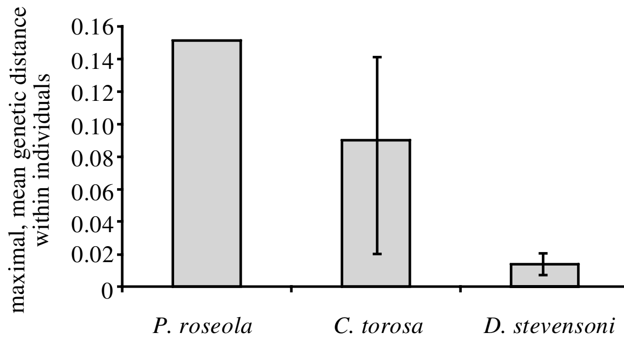

Figure 2. Maximal genetic distance (HKY 85; Hasegawa *et al.* 1985) of *hsp82* within individual genomes. For *Darwinula stevensoni* and *Cyprideis torosa*, individuals with maximal means of genetic divergence are shown. Error bars show 95% CIs of means. Distances for bdelloid rotifers were recalculated from GenBank sequences (Mark Welch & Meselson 2000) of which *Philodina roseola* showed maximal individual divergence.

described for humans (Clark 1996). Lower levels of metabolic activity and/or fewer cell divisions in embryos compared with adults could also have contributed to the observed, significant difference in genetic diversity between the age classes.

The exceptionally low genetic variability in the single copy gene(s) at all levels, even the interpopulation comparisons, shows that there is no apparent Meselson effect in *D. stevensoni*. This becomes even more clear if maximal genetic diversities within individual ostracods and bdelloid rotifers are compared directly (figure 2). Owing to the asymmetry of the test (Butlin 2000, 2002), however, this does not imply that *D. stevensoni* is not an ancient asexual, as several mechanisms could counter the accumulation of mutations.

Haploidy could be an elegant way to avoid the accumulation of mutations for millions of years, because first, it makes all slightly deleterious mutations pseudodominant, thereby increasing the efficiency of purifying selection (Lynch *et al.* 1993). Second, haploidy reduces the number of gene loci that can mutate, especially in combination with a large population size (Haigh 1978; Lynch *et al.* 1993).

It is theoretically possible that *D. stevensoni* is a haploid organism, although karyologic screening falsifies this; Tétart (1978) found 22 chromosomes in *D. stevensoni*, which is perfectly in line with numbers from other diploid, non-marine ostracods. This would also be only the second case of a female haploid in a metazoan. The only known example, a female haploid mite, is special, as it orginated from haploid males that got infected with a feminizing, endosymbiotic bacterium (Weeks *et al.* 2001). The Meselson effect could also have been so strong that second alleles have deteriorated beyond recognition. In the latter case, the species would be a functional haploid and no primers would amplify this second allele. Unless future experiments provide evidence for haploidy, this possibility must be rejected, because embryos show some genetic divergence. The observed genetic uniformity, in fact almost identity, in nuclear sequences of geographically widely separated populations provides further evidence against haploidy.

This genetic pattern cannot have been caused by a recent selective sweep, as darwinulids are poor dispersers (they are the only ostracods without dry-resistant eggs). Also, some genetic divergence was observed in a mitochondrial gene and no speed-up in molecular rates of mitochondrial evolution has been found (see Schön *et al.* (1998) for more details), not even within the family of Darwinulidae as a whole (Schön *et al.* 2003). European and African populations of *D. stevensoni* have been separated for an estimated 7.6–4.4 Myr (Schön *et al.* 1998) and haploid, nuclear sequences should have accumulated a significant amount of genetic change, but this is not the case.

The absence of the Meselson effect in *D. stevensoni* can also not be explained by rare syngamy nor by cryptic sex, as permutation likelihood tests identify recombination (with genetic exchange) as a possible explanation for one out of 21 instances in *hsp82* only. Most likely, this single deviation is due to a technical artefact such as PCR jumping or PCR recombination (Pääbo *et al.* 1990; Bradley & Hillis 1997). In the apparent absence of rare or cryptic sex, other homogenizing mechanisms need to be identified. These should also be more efficient than sex in the removal of genetic changes considering that genetic divergence within individuals of *D. stevensoni* is significantly lower than within the sexual ostracod *C. torosa* (figure 2) and genetic divergence is low between populations. Such potential mechanisms have been suggested.

Gene conversion between alleles can effectively homogenize genomic regions (Rourke & East 1997; Butlin 2000, 2002). In *D. stevensoni*, the modified permutation likelihood tests for gene conversion (McVean *et al.* 2002) give significant departures for the multiple ITS region only (table 2). Homogenization of this region should act on both alleles and copies effectively causing concerted evolution. Most documented cases of gene conversion (15 out of 17; see electronic Appendix A available on The Royal Society's Publications Web site) so far come from gene families (Charles *et al.* 1997; Rourke & East 1997; Wang *et al.* 1999; Lazzaro & Clark 2001) or multi-copy regions like rRNA clusters (Benevolenskaya *et al.* 1997) and ITS (Fuertes Aguilar *et al.* 1999). Data from well-studied organisms such as yeast, *Drosophila* and *Arabidopsis* suggest that gene conversion can occur anywhere in the genome (e.g. Bertrán *et al.* 1997; Abdullah & Borts 2001; Langley *et al.* 2001; Haubold *et al.* 2002). Thus, although gene conversion is known to happen in single copy genes through repair of double strand breaks (Osman & Subramani 1998; Johnson & Jasin 2000), no evidence of this has been found in *D. stevensoni*. No gene conversion has as yet been detected in bdelloid rotifers (see Butlin 2000; Mark Welch & Meselson 2000), but only single copy genes have thus far been analysed in this group.

Although screening of mother–daughter pedigrees in non-marine, asexual ostracods has so far only confirmed apomixis as the asexual reproductive mode (e.g. Havel & Hebert 1989; Chaplin *et al.* 1994), the occurrence of automixis in *D. stevensoni* cannot at the moment be ruled out as a potential explanation for the low genetic diversity. However, only one out of three types of automixis does effectively increase homozygosity, when diploidy is restored by terminal fusion of two haploid products after the second meiotic division (Butlin *et al.* 1998; Maynard

Smith 1998). This type of automixis still implies recombination (Butlin *et al.* 1998), which, because of the increase in homozygosity, will not be detectable within but only between individuals. The permutation likelihood tests applied here (see above) do not signal the presence of such recombination. Moreover, even automixis could not account for the exceptionally low genetic distances between populations of this species. Alternative mechanisms must therefore be sought to explain the overall homogeneity of the genome in *D. stevensoni*.

Highly efficient DNA repair (Schön & Martens 1998) is one possible mechanism. If chemical changes in DNA are actively repaired before they become manifested as mutations, a low genetic divergence within individuals will result. The higher genetic variability in a mitochondrial gene (COI) of *D. stevensoni* (Schön & Martens 1998; Schön *et al.* 1998) hints at such a mechanism as DNA repair in mitochondrial DNA being less efficient than in nuclear DNA (LeDoux *et al.* 1992). The presented results indicate that such efficient DNA repair would also operate in germ line cells.

Speculation aside, we have shown that *D. stevensoni*, which has survived for millions of years in the complete absence of sex, prevented the accumulation of mutations. If highly efficient DNA repair or other, special genetic mechanisms have been selected for to overcome the negative effects of the mutational load hypothesis and Muller's ratchet, then our data support the validity of these hypotheses to explain the prevalence of sex in the animal and plant kingdoms (Maynard Smith 1978). Earlier data on the bdelloids, however, demonstrate that such accumulations can be handled without deleterious effects over long time-frames (Mark Welch & Meselson 2000), even if the molecular rates of evolution appear unaffected (Mark Welch & Meselson 2001). However, in the absence of special genetic mechanisms, the bdelloids have adapted alternative ecological strategies, such as large population size and very short life cycles of a few days only. Both extant ancient asexual animal groups have thus persisted without sex for millions of years, albeit with opposite evolutionary strategies. It shows that at least these groups are no slaves to sex (Dagg 2000).

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