

Evidence for Inefficient Selection Against Deleterious Mutations in Cytochrome Oxidase I of Asexual Bdelloid Rotifers

Timothy G. Barraclough,*†‡ Diego Fontaneto,*‡ Claudia Ricci,‡ and Elisabeth A. Herniou*

*Division of Biology and the NERC Centre for Population Biology, Imperial College London, Berkshire, United Kingdom;

†The Jodrell Laboratory, Royal Botanic Gardens, Kew, United Kingdom; and ‡Università di Milano, Dipartimento di Biologia, Milano, Italy

Evolutionary theory predicts that natural selection should be less efficient in asexually than in sexually reproducing organisms. Obligate asexuals are expected to adapt slowly to changing environments and to accumulate mildly deleterious mutations to their genomes, potentially explaining their typically short evolutionary lifespans. One group of animals that appear to challenge these ideas is the bdelloid rotifers, a large and ancient clade of obligate asexuals. Previous work has found no evidence for inefficient selection against deleterious mutations in protein-coding genes of bdelloids. However, these studies relied mostly on between-species comparisons and were therefore unable to detect mildly deleterious mutations that persist within populations but are removed by selection over longer time periods. Here, we test for inefficient purifying selection acting on the cytochrome oxidase I (*coxI*) mitochondrial gene in 3 clades of bdelloids. Patterns of variation are compared to those of two facultatively sexual clades: a monogonont rotifer (*Brachionus*) and a branchiopod crustacean (*Daphnia*). As predicted due to the strict linkage between mitochondrial and nuclear genomes, bdelloids exhibit higher frequencies of putatively deleterious amino acid polymorphism within populations than the two facultatively sexual clades. While the monophyly and age of bdelloids makes it hard to rule out other explanations for the observed differences, several possible confounding factors, such as differences in effective population size or patterns of codon usage, are shown not to explain the observed differences. We therefore conclude that bdelloid mitochondrial DNA variation does display the signature of inefficient selection expected of obligate asexuals.

Introduction

Most multicellular organisms are sexual—their life-cycle includes a stage at which genes from different individuals recombine to produce genetically mixed progeny (Bell 1982). Obligate asexuality, in which individuals inherit all their genes from a single individual by apomixis, has evolved quite frequently in some clades (Mogie 1992; Butlin et al. 1998). However, most extant asexuals evolved recently, consistent with the idea that asexuals tend to be evolutionary dead-ends (Maynard Smith and Szathmari 1995).

One explanation for the rapid demise of most asexuals is that strict linkage reduces the efficiency of natural selection (Weismann 1904; Burt 2000). Beneficial and deleterious mutations can spread and dwindle in a sexual population without interference from selection acting on unlinked loci. In contrast, in asexual populations the fate of individual mutations is tied strictly to the entire genetic background. As a consequence, asexual populations are expected both to adapt slowly to changing environments (Otto and Lenormand 2002; Goddard et al. 2005) and to accumulate mildly deleterious mutations that would have been lost from a recombining population (Kondrashov 1988; Lynch et al. 1993; Gordo and Charlesworth 2000; Keightley and Eyre-Walker 2000). Both aspects could contribute to the high extinction risk of asexual species.

Here we test for inefficient natural selection in a group of animals that presents a major challenge to conventional theories of sex, the bdelloid rotifers. Bdelloids are abundant microscopic animals found in permanently or occasionally wet habitats such as lakes, streams, soil, moss and lichen (Donner 1965; Ricci 1987). No males or traces of meiosis

have ever been observed and molecular evidence supports their long-term asexuality: nuclear genes found as a pair of similar copies (alleles) in diploid sexuals are present only as divergent copies in bdelloids, consistent with the absence or extreme rarity of sexual reproduction, perhaps for 100 million years (Mark Welch and Meselson 2000; Birky 2004; Mark Welch et al. 2004; Mark Welch et al. 2004). Other unusual features of their genomes offer further support for their obligate asexual status (Arkhipova and Meselson 2000). Yet, bdelloids comprise around 460 described species taxa (Segers 2007), making it the largest clade of obligate asexual plants or animals (Butlin 2002).

As famously noted by John Maynard Smith, bdelloids pose a challenge to theories on the evolution of sex (Maynard Smith 1992). How have they managed to survive and prosper over long evolutionary timescales without the presumed advantages of a sexual lifestyle? One possibility is that they simply bear the costs of asexuality, perhaps mitigated by their lifestyle (Ricci 1987) or because the costs are less than widely supposed (Keightley and Eyre-Walker 2000). Alternatively, bdelloids might have special mechanisms to avoid the costs of asexuality, for example extremely efficient DNA repair mechanisms or some unknown mechanism of cryptic or very rare recombination (Birky 2004).

We test for the accumulation of deleterious mutations in the mitochondrial gene cytochrome oxidase I (*coxI*) in bdelloids, predicted to occur if bdelloids bear the costs of an asexual lifestyle. Although mitochondrial genes are always strictly linked to one another, in asexual lineages they are linked to the entire nuclear genome, hence theory predicts far stronger selective interference affecting mitochondrial genes in obligate asexuals than in sexuals (Birky et al. 2005; Paland and Lynch 2006a). Our analyses assume that silent mutations are effectively neutral and that *coxI* is under generally purifying selection, such that most amino acid mutations are deleterious. This assumption is supported by a very low ratio of amino acid to silent substitutions, Ka/Ks (ratio of amino acid changes to silent changes) (Results,

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E-mail: t.barraclough@imperial.ac.uk.

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Birky et al. 2005). Under these conditions, if selection is indeed less efficient at removing deleterious amino acid mutations in asexual lineages, then asexuals should display higher Ka/Ks ratios than those observed in comparable sexual groups. This prediction was recently confirmed in comparisons between sexual and asexual *Daphnia pulex* (Paland and Lynch 2006a).

Two previous studies have compared Ka/Ks ratios between bdelloid rotifers and their nearest comparable sexual relatives, facultatively sexual monogonont rotifers. One used the *cox1* gene (Birky et al. 2005) and one the nuclear heat shock protein *hsp82* gene (Mark Welch and Meselson 2001). Both found no consistent difference between the 2 clades and concluded that bdelloids show no evidence of having less efficient selection than the monogononts. However, as discussed by Birky et al. (2005), both were based on low sample sizes and mainly (entirely in the case of *hsp82*) on comparisons between different species. As well as limiting the power of comparisons, such samples permit comparison of only 1 class of mutations, namely those with weak enough effects to become fixed and persist over long evolutionary timescales (Birky et al. 2005). By sampling both within and between populations, we can also estimate the frequency of mildly deleterious mutations that persist as polymorphism within populations but tend to be removed by selection over longer periods (Smith and Eyre-Walker 2002; Paland and Lynch 2006a). The relative effect of inefficient selection on the 2 classes of mutations will depend on the frequency distribution of mutational effects, an unknown quantity here. However, if most mutations contributing to polymorphism and divergence are mildly deleterious, the effects should be most apparent in the latter class (Weinreich and Rand 2000). Note that both classes of mutation will influence the mean fitness of asexual populations: fixation is not necessary to impart a cost of asexuality.

We sequenced *cox1* from samples of individuals from 3 commonly encountered genera of bdelloids, *Adineta*, *Philodina* and *Rotaria*. Sample sites were distributed globally but focused on Italy and the UK. Because of the potentially wide dispersal of these taxa and the presence of numerous cryptic taxa revealed by genetic studies (Fontaneto et al. 2007a), we use the sequence data themselves to identify genetic clusters for use as “population” units in subsequent analyses, following the approach of Pons et al. (2006) and Fontaneto et al. (2007a). Ka/Ks ratios were then estimated both within and between genetic clusters to test for excess amino acid polymorphism within clusters, following the rationale of McDonald and Kreitman (1991).

The age and monophyly of bdelloids prevent replicate comparisons with sexual lineages, for example, as performed by Paland and Lynch (2006a) for *D. pulex*, and means that only comparisons with distant relatives are possible. However, we chose 2 broadly comparable sexual clades to provide a benchmark for the bdelloid data. First, we use the dataset of Gómez et al. (2002) for monogonont rotifers of the *Brachionus plicatilis* species complex. Monogononts are the nearest comparable sexual relatives of bdelloids. They are microscopic, facultatively sexual, but also differ from bdelloids in being more strictly aquatic

and by tolerating drought as resting eggs, rather than through anhydrobiosis (= dormancy induced by desiccation, Ricci 1987; Cáceres 1997; Ricci 2001). Mixis has been observed directly in several of the sampled *B. plicatilis* populations and is assumed to occur in the others (Serra, Gómez, and Carmona 1998; Gómez et al. 2002).

Other related taxa to bdelloids such as the seasonid rotifers, numbering just three species living externally as an epibiont on marine crustacea, or acanthocephalans, which are macroscopic parasites of invertebrate and vertebrate hosts, have very different lifestyles that might confound comparison (Sørensen and Giribet 2006). Therefore, as a second clade of facultative sexuals for comparison we used published data from *Daphnia*. *Daphnia* are tiny to microscopic, aquatic crustacea, distantly related to rotifers but overlapping in habitats with several rotifers, and have been widely used for studies of obligate and facultative asexuality (Little and Ebert 2000; Duncan, Mitchell, and Little 2006). The genus includes a few species that are obligate asexuals (Hebert 1981) and some with both obligate asexual and facultatively sexual populations (Hebert, Ward, and Weider 1988). The sample studied here does not include multiple individuals for any species believed to comprise just obligate asexuals; therefore we assume that variation in all the sampled species evolved in the presence of recombination.

Our analyses show that bdelloids do indeed bear the expected signature of inefficient selection due to obligate asexuality. Bdelloid clusters harbor levels of amino acid polymorphism between 2 to 30 times higher than those found in the facultatively sexual clusters. Although we cannot rule out causes for the pattern other than sexuality, population genetic measures of several possible confounding factors, such as population size and past demography, are shown not to vary consistently between bdelloids and the other clades.

Materials and Methods

Sampling

We collected all individuals of the genera *Rotaria* and *Adineta*, and of the species complex *Philodina flaviceps*, encountered during 3 years searching rivers, standing water, dry mosses and lichens. Most samples were obtained in Italy and the UK but also globally as part of a wider study of bdelloids (Fontaneto et al. 2007a; Fontaneto et al. 2007b). These clades were chosen because of ease of identification, because they are commonly encountered in suitable habitat and because they are monophyletic in broader analyses of bdelloid *cox1* variation (Herniou and Barraclough, unpublished data). *Adineta* tends to be found in drier habitats, whereas *P. flaviceps* and *Rotaria* are mostly found in fully aquatic habitats such as ponds, streams and bogs. Outgroups were included as follows: for *Adineta*, two individuals from the related genus *Bradyscelia*; for *P. flaviceps*, one individual of *P. acuticornis*, two of *P. rugosa* and five of *P. citrina*; for *Rotaria*, eight individuals from the related genus *Dissotrocha*. DNA was isolated either from clonal samples of 5 to 25 individuals grown in the lab from a single wild-caught individual or from single wild-caught

individuals using a chelex preparation (InstaGene Matrix, Bio-Rad) as per manufacturers instruction with volumes varying from 15 to 50 μ l depending of the number of animals used.

DNA Amplification and Sequencing

Cytochrome oxidase I (*cox1*) was PCR amplified using optimized primers Hcox1 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and Lcox1 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'). Cycle conditions comprised initial denaturing at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 40°C for 1 minute and 72°C for 90 seconds, and a final extension step of 72°C for 5 minutes. An annealing temperature of 43°C or 46°C was used for the *P. flaviceps* reactions. Cycle sequencing reactions were set up using PCR primers and the ABI Big Dye Terminator v1.1 kit and run on an ABI 3770 automated sequencer. The sequences were checked and assembled using Sequencher 4.1 (Gene Code Corporation), aligned with ClustalW and edited by eye in MacClade 4.0. The final alignment includes newly reported sequences for *Adineta* and *Rotaria* (EF173178–EF173270), together with previously reported sequences for *Adineta* (AY218092–AY218093, Giribet et al. 2004, DQ078512–DQ078514, DQ078516 and DQ078519, Birky et al. 2005), *Rotaria* (DQ656756–DQ656882, Fontaneto et al. 2007a, AY218094, Giribet et al. 2004) and *P. flaviceps* (DQ890071–DQ890156, Fontaneto et al. In Revision). Locality data for newly reported sequences are provided as a supplementary table.

Sequence Retrieval

We downloaded *cox1* mtDNA sequences from Genbank for the monogonont *B. plicatilis* species complex and for *Daphnia*. *Brachionus* sequences were those from the study by Gómez et al. (2002), chosen because it represented a similar scale of study with similar sampling strategy: focused in Spain but including worldwide samples. Fifteen sequences from *Keratella* species, 2 from *B. calyciflorus* and 1 from *B. quadridentatus* were included as outgroups. The *Daphnia* sequences were downloaded using the search term “Daphnia and (COI or COX)” in Entrez. A sequence for *Scapholeberis sp.* was used as an outgroup. Because of the greater intensity of sampling for *Daphnia* than the other clades, the dataset was rarefied by randomly choosing 10 sequences for any Latin binomial represented by more than 10 sequences, using a routine in the R statistical programming language (R Development Core Team 2006). Sequences were aligned and edited as indicated above. Aligned matrices for all the study clades are available from T.G.B.

Phylogenetic Analyses

Trees were reconstructed by Bayesian analysis run in MrBayes 3.1.2 for 5 million generations with 2 parallel searches, using a GTR +invgamma model (Ronquist and

Huelsenbeck 2003). This substitution model was indicated as significantly preferred over simpler ones by MrModelTest (Nylander, 2004). The consensus tree showing all compatible groupings was used as the input tree for subsequent analyses. Trees were rooted using the outgroups. Ultrametric trees required for the clustering model were obtained by rate smoothing the consensus tree for each clade using penalized likelihood in r8s and cross-validation to choose the optimal smoothing parameter (Sanderson 2002).

Identifying Units for Comparison

We used the branching rate models of Pons et al. (2006) to identify genetic clusters of very closely related individuals separated by longer internal branches from other such clusters, representing distinct populations or species. Under a null model that the entire sample for each study clade derives from a single population obeying a single coalescent process, i.e., no clusters are present, we calculated the likelihood of waiting times, x_i , between successive branching events on the DNA tree as:

$$L(x_i) = b * e^{-b*x_i} \quad (1)$$

where

$$b^* = \lambda(n_i(n_i - 1))^p \quad (2)$$

where n_i is the number of lineages in waiting interval i , λ is the branching rate for the coalescent (the inverse of twice the effective population size in a neutral coalescent), and p is a scaling parameter that allows the apparent rate of branching to increase or decrease through time, fitting a range of qualitative departures from the strict assumptions of a neutral coalescent, for example growing ($p < 1$) or declining ($p > 1$) population size (Pons et al. 2006).

Under the alternative model that the sample derives from a set of independently evolving populations, each one evolving similarly to the null case, we calculated the likelihood of waiting times as equation 6 from Pons et al. (2006). The alternative model optimizes a threshold age, T , such that nodes before the threshold are considered to be diversification events with branching rate λ_D and scaling parameter p_D . Branches crossing the threshold define k genetic clusters each obeying a separate coalescent process but with branching rate, λ_C , and scaling parameter, p_C , assumed to be constant across clusters. The alternative model thus has three additional parameters. Models were fitted to the ultrametric trees for each study clade using an R script available from T.G.B.

Estimation of Frequency of Amino Acid Changes

We assumed that silent mutations are effectively neutral and that *cox1* is under generally purifying selection, such that most amino acid mutations are deleterious. Using ω to denote the Ka/Ks ratio, ω_{intra} the Ka/Ks ratio within clusters and ω_{inter} the Ka/Ks ratio between clusters, we can estimate the frequency of mutations that fall into different classes. The frequency of strongly deleterious mutations that are removed over short timescales and never found within populations is $1 - \omega_{intra}$. The frequency of

Table 1
Sample Sizes and Numbers of Clusters for Each of the 5 Study Clades

Class	Clade	Length of aligned sequence	Number of individuals	Number of clusters	Mean number of individuals per cluster ^a
Bdelloidea	<i>Adineta</i>	657	88	19	3.7
	<i>P. flaviceps</i>	618	86	8	10.5
	<i>Rotaria</i>	657	77	17	3.4
Monogononta	<i>Brachionus</i>	588	53	9	4.9
Branchiopoda	<i>Daphnia</i>	609	120	18	4.8

^a Calculated as the total number of individuals belonging to clusters of 2 or more individuals, divided by the total number of clusters.

mildly deleterious mutations found within populations but removed over longer timescales is $\omega_{\text{intra}} - \omega_{\text{inter}}$ (Paland and Lynch 2006a). Finally, the frequency of nearly neutral mutations that persist and become fixed between distantly related individuals is ω_{inter} . The latter category would also contain any adaptive substitutions between clusters but these are assumed to be negligible both within and between clusters: the effects of deviations from this assumption are discussed below.

Values were estimated using branch-class models in PAML (Yang 1997), labeling branches depending on whether they are optimized to be within-cluster or between-cluster branches by the clustering models. The likelihoods of 2-parameter models, optimizing different ω_{intra} and ω_{inter} , for each clade were compared to those with a single average ω for the entire tree using log likelihood ratio tests. The consensus tree (all compatible) from the Bayesian analysis was used as the input tree. To check the robustness of the results, we repeated rate-smoothing, clustering analysis and Ka/Ks estimation on 10 randomly chosen trees from the MCMC chains for each clade. Optimization in PAML was too slow to allow comparison of more sampled trees, but 10 proved sufficient to judge variability (see Results). Because of constraints on processor time, we only fitted the alternative model to these trees and compared Ka/Ks estimates with those obtained from the consensus tree. PAML estimations for these 50 trees were run in parallel on the Opteron based beowulf cluster hosted by the London E-Science Centre.

Population Genetic Parameters

We used DNAsp to estimate a number of population genetic signatures relating to population size, demographic history and the history of selection (Rozas et al. 2003). First, we estimated theta, which represents the product of effective population size and mutation rate. Next, we estimated 3 related statistics designed for detecting the history of population growth or various forms of selection. Fu and Li's F^* statistic was introduced as a general statistic for detecting purifying selection against deleterious mutations or a recent selective sweep or hitch-hiking event (Fu and Li 1993), but subsequently shown to be most powerful for detecting purifying selection (Fu 1997). Fu's F_S statistic and Ramos-Onsins and Rozas's R_2 statistic offer strongest power for detecting population growth or a recent selective sweep (Fu 1997; Ramos-Onsins and Rozas 2002), both of which represent a scenario of recent increase in the numbers of

gene copies from a common ancestor. Because bdelloid mtDNA is linked to the entire genome, we might predict a stronger signature of both purifying selection and selective sweeps in bdelloids than in the other clades: for example, every selective sweep in bdelloids should affect all loci (Birky et al. 2005). However, all 3 statistics are related, testing for an excess of mutations on recent branches of the genealogy in slightly different ways, and therefore all are potentially influenced by interacting processes of demography and selection. Demographic differences among clades could obscure any predicted difference due to the effects of selection on strictly linked genomes.

The significance of F^* , F_S and R_2 for each cluster was assessed against simulations in DNAsp assuming a neutral coalescent for a constant sized population and the empirical estimates of theta. A combined probability of departure from neutral expectations for all clusters within each clade in turn was calculated using a weighted Z-test (Whitlock 2005). We next used analysis of variance to test for significant variation in the average values of each statistic among the 5 clades and Tukey's Honestly Significant Difference test to interpret which clades differed in mean values.

Codon Bias

Our analyses assume that silent mutations are effectively neutral. This may not be the case if selection favors certain codons over others for a given amino acid, for example a bias in base composition (Butlin 2006; Paland and Lynch 2006b). Therefore, we estimated the base composition of silent sites in DNAsp and compared mean base composition between the 5 study clades and among clusters within each clade. Note that in order to influence our within- and between-cluster comparisons, it would be necessary for the optimum base composition to vary between clusters within clades to varying degrees, not just for there to be differences in optimum base composition among the 5 study clades.

Results

Summary statistics for the compiled data are provided in table 1. Bayesian phylogenetic analysis revealed generally high support for more recent branching events, but lower support towards the base of the trees (Supplementary Material). Trees tended to recover most traditional taxonomic species as monophyletic in *Rotaria* and *Daphnia*

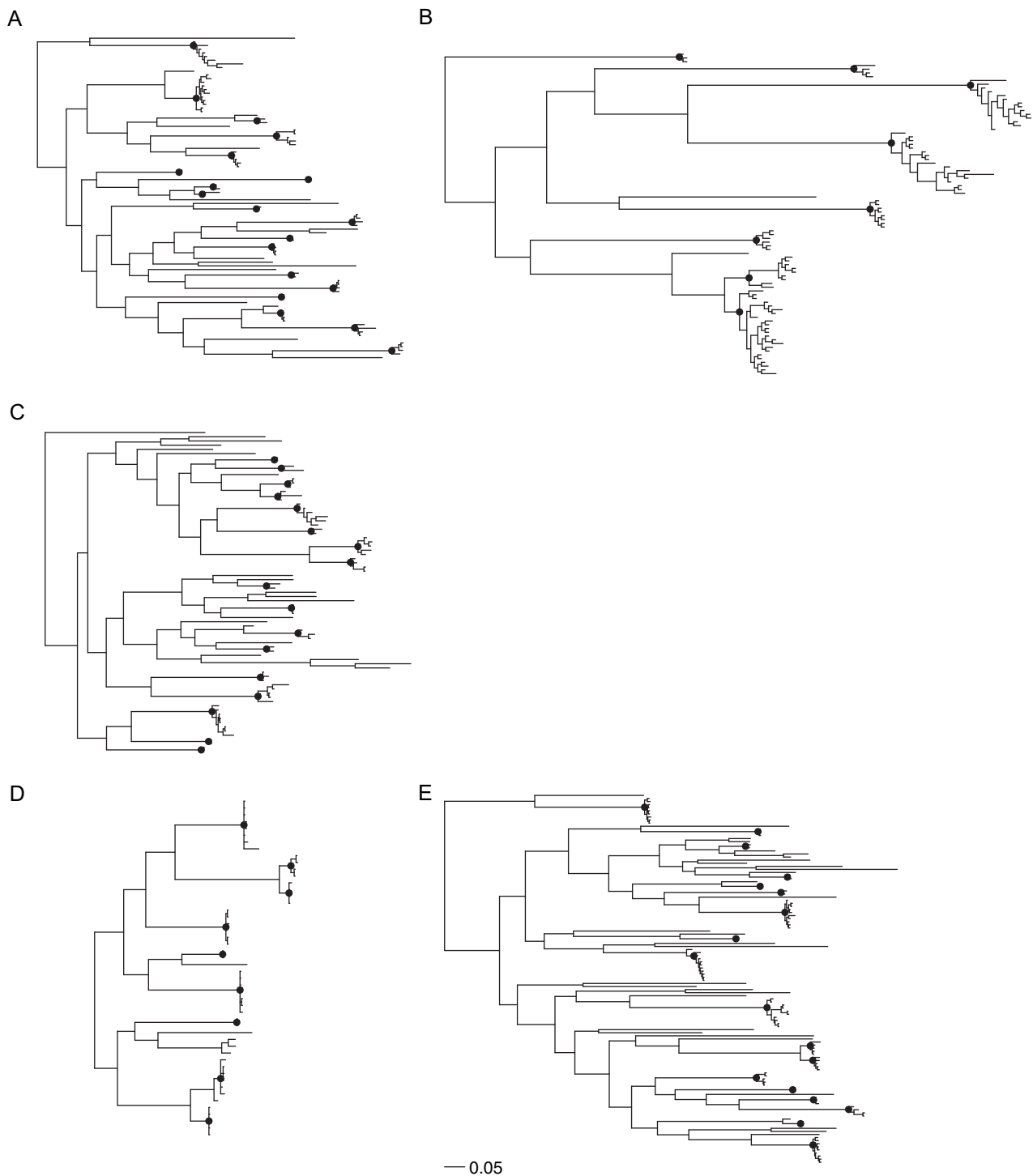


FIG. 1.—The consensus trees showing all compatible groupings from Bayesian analyses of *cox1* from the 5 study clades. (A) *Adineta*; (B) *P. flaviceps*; (C) *Rotaria*; (D) *B. plicatilis*; (E) *Daphnia*. Black dots indicate branches defining genetic clusters identified by the branching rate models. All 5 phylograms are drawn to scale: the scale bar indicates 0.05 substitutions per site. Trees with sample names and Bayesian support values are provided as Supplementary Information.

(Fontaneto et al. 2007a, Supplementary figures 3 and 5). The sample of *Adineta* included a few traditional species but the sequence data reveal massive levels of previously undescribed diversity, not unexpected based on traditional systematic treatments of the group (Donner 1965). The likelihood analyses of branching rates detected significant clus-

tering in all of the clades ranging from 8 clusters in *P. flaviceps* to 19 clusters in *Adineta* (table 1, fig. 1). In all cases, the null model of no clusters being present could be rejected (log likelihood ratio tests, all chi-square ≥ 38.8 , 3 degrees of freedom, all $p << 0.001$). Clusters in the different clades had very similar properties in terms

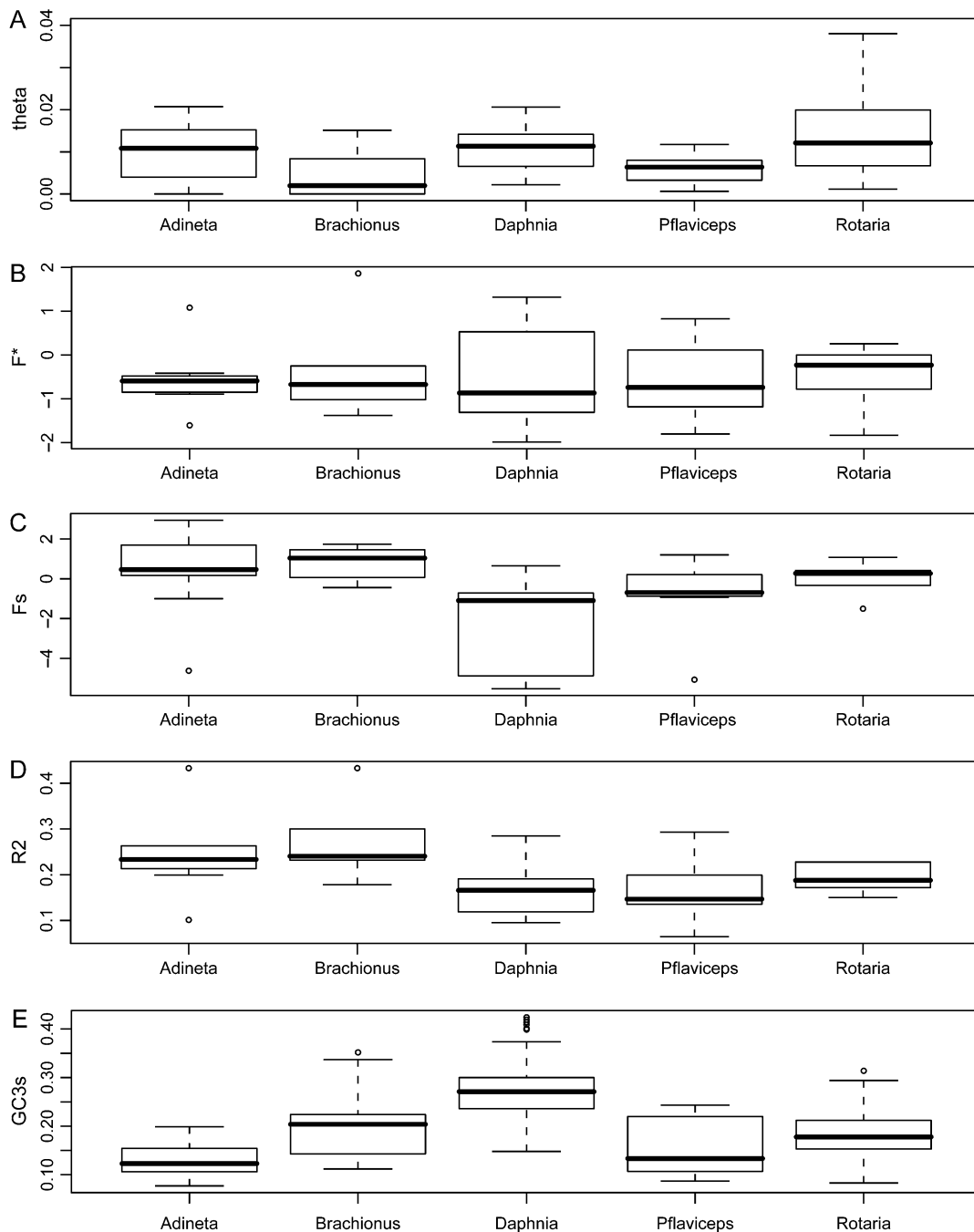


FIG. 2.—Box-and-whisker plots showing the median value of the 4 population genetic measures estimated across clusters within each clades. Boxes indicate the extent of the first and third quartiles. Whiskers indicate the most extreme data point not more than 1.5 times the inter-quartile distance away from the box. (A) Theta; (B) F^* ; (C) F_S ; (D) R_2 ; (E) GC3s = frequency of C or G at silent third positions.

of average amounts of within cluster genetic variation: not necessarily the case *a priori* since the approach fits a separate threshold for defining clusters in the different clades. The median of the average pairwise genetic distance within clusters ranged from 0.2% in *Brachionus* to 1.2% in *Rotaria* (fig. 2A).

Fitting a model with a single ω across the entire tree confirms that *cox1* is under generally purifying selection in all 5 clades. Values of ω ranged from 0.0015 in *Brachionus* to 0.012 in *Rotaria* (table 2), translating into selective con-

straints, $1-\omega$, ranging from 0.988 to 0.999 (Paland and Lynch 2006). In addition, all of the study clades show an excess of amino acid polymorphism within clusters relative to divergence between clusters (fig. 3A), significant in all clades except *Brachionus* (table 2). This pattern is consistent with the presence of mildly deleterious mutations within populations that are removed by selection over the longer term. The average frequency of such mutations in each clade, $\omega_{\text{intra}}-\omega_{\text{inter}}$, is higher in the bdelloid clades than in *B. plicatilis* or *Daphnia* (fig. 3B). The bdelloid

Table 2
Maximum Likelihood Estimates of Ka/Ks Ratio and Model Comparisons using the Bayesian Consensus Tree

	Null model ^a	Alternative model		χ^2	<i>p</i>
	Global ω	ω_{intra}	ω_{inter}		
<i>Adineta</i>	0.0075 ± 0.0007	0.038 ± 0.006	0.0052 ± 0.0006	81.7	<<0.001
<i>P. flaviceps</i>	0.0086 ± 0.0015	0.099 ± 0.022	0.0017 ± 0.0006	125.7	<<0.001
<i>Rotaria</i>	0.0119 ± 0.0009	0.057 ± 0.008	0.0083 ± 0.0008	100.7	<<0.001
<i>Brachionus</i>	0.0015 ± 0.0004	0.004 ± 0.003	0.0013 ± 0.0004	1.8	>0.100
<i>Daphnia</i>	0.0039 ± 0.0005	0.020 ± 0.006	0.0033 ± 0.0004	22.5	<<0.001

^a Standard errors estimated by the curvature method in PAML are shown.

clades have frequencies of mildly deleterious mutations that range from twice to over 30 times as great as those in *B. plicatilis* or *Daphnia*. Standard errors in the estimates across Bayesian trees are extremely low (table 3), indicating that the result is robust to uncertainty among sampled trees and to the use of the consensus tree rather than individual sampled trees. Although the numbers of sampled individuals per cluster varies among the study clades, which might affect the accuracy of estimates of ω_{intra} and ω_{inter} , it does not vary systematically with the estimated Ka/Ks ratios, for example *P. flaviceps* has the highest sample of individuals per cluster, followed by *B. plicatilis* and *Daphnia*.

To verify the significance of the difference in ω_{intra} – ω_{inter} between bdelloids and the other clades, we performed an approximate likelihood ratio test comparing a model with a single ω_{intra} constrained across all 5 clades (the null model) to a model with two ω_{intra} parameters: one for the bdelloids and one for the other two clades. The test is, of necessity, approximate, because the software does not allow joint estimation of parameters across the 5 clades and does not allow both ω_{intra} and ω_{inter} to be constrained. We chose to constrain ω_{intra} because variation in this parameter is the main cause of observed variation in ω_{intra} – ω_{inter} (fig. 3). In the null model, ω_{intra} was constrained to the weighted mean ω_{intra} across the 5 clades when optimized separately (0.0489), weighting by the number of within-cluster branches in each clade. In the alternative model, ω_{intra} was constrained in the bdelloids to the weighted means across the 3 bdelloid clades (0.0701) and in the other 2 clades (0.0146). The constrained model with a different ω_{intra} in bdelloids versus the other 2 clades is significantly more likely than the null model with a single constrained ω_{intra} (twice the difference in the sum of log likelihoods, $\chi^2 = 13.0$, $p < 0.001$). Therefore, bdelloids do have significantly higher ω_{intra} – ω_{inter} than the 2 facultatively sexual clades.

The population genetic statistics relating to population size, population growth, and the history of selection varied inconsistently between bdelloids and the other clades (fig. 2). Theta varies significantly among the clades (anova, $F_{4,65} = 4.1$, $p = 0.0052$), ranging from an average of 0.0044 in *Brachionus* to 0.015 in *Rotaria*. However, Tukey's Honestly Significant Difference test revealed that only the comparison between *Rotaria* and *Brachionus* was strongly significant ($p = 0.0054$) and the comparison between *Rotaria* and *P. flaviceps* marginally significant ($p = 0.046$): *Rotaria* has marginally higher values of theta than the other clades.

F^* did not vary significantly among clades (anova, $F_{4,31} = 0.05$, $p > 0.9$). Note that the residual degrees of

freedom are lower because F^* and subsequent statistics were only calculated for clusters with 4 or more sequences sampled. F^* was significantly negative across clusters in *P. flaviceps* compared to the neutral simulations in DNAsp (Z-test combining separate simulated *p*-values for each cluster, $Z = -2.71$, $p = 0.0034$), and negative but not significant in the other clades (fig. 2).

F_S did vary significantly among clades, with *Daphnia* clusters displaying lower scores than the other clades

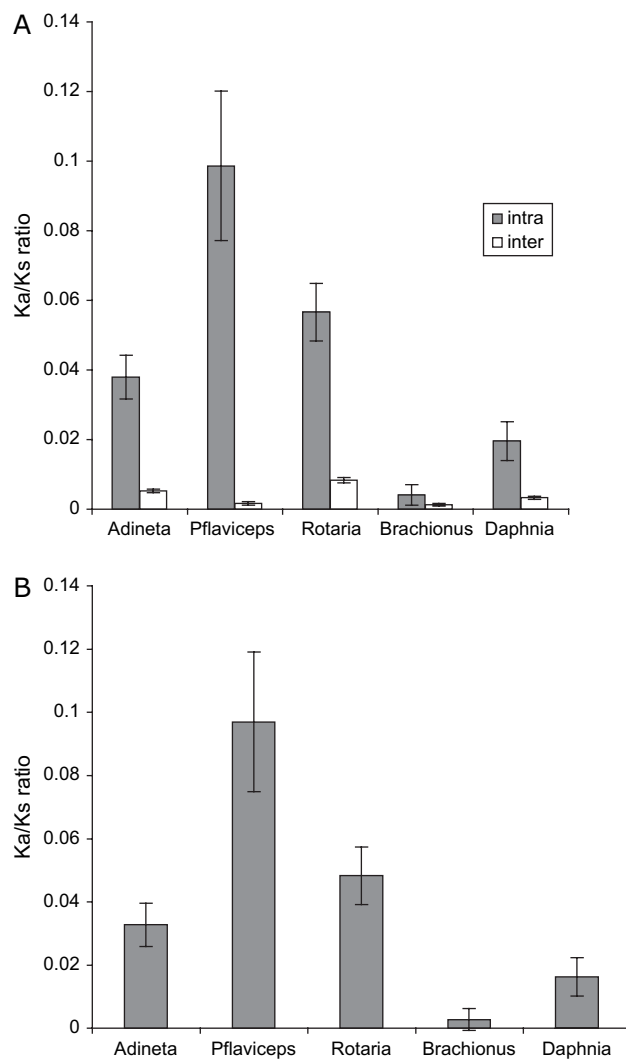


FIG. 3.—(A) Mean and standard errors of estimates of ω_{intra} and ω_{inter} . (B) Mean and standard errors of $\omega_{\text{intra}} - \omega_{\text{inter}}$.

Table 3
Mean and Standard Error of Estimates of Ka/Ks Ratio
Across 10 Randomly Chosen Bayesian Trees for Each Clade

	Alternative model	
	ω_{intra}	ω_{inter}
<i>Adineta</i>	0.040 ± 0.0011	0.0051 ± 0.00004
<i>P. flaviceps</i>	0.089 ± 0.0010	0.0018 ± 0.00007
<i>Rotaria</i>	0.058 ± 0.0012	0.0084 ± 0.00003
<i>Brachionus</i>	0.004 ± 0.0001	0.0013 ± 0.00002
<i>Daphnia</i>	0.016 ± 0.0003	0.0033 ± 0.00001

(anova, $F_{4,31} = 3.4$, $p = 0.019$), marginally significant in the case of the comparisons with *Adineta* and *Brachionus* (Tukey's HSD test, $p = 0.036$ and 0.029 respectively). R_2 also varied marginally significantly among clades (anova, $F_{4,31} = 2.9$, $p = 0.037$), although none of the pairwise comparisons of clades were significantly different (Tukey's HSD test). Both *P. flaviceps* and *Daphnia* displayed significantly lower F_S and R_2 scores than expected from the neutral coalescent and constant population size simulations (combined Z-tests, all $Z < -2.76$, all $p < 0.003$).

Codon usage varies significantly among the study clades (anova, $F_{4,31} = 105.4$, $p < 0.001$, fig. 2). The mean frequency of C or G usage at silent third positions ranged from 13.1% in *Adineta* to 27.8% in *Daphnia*. The bdelloid clades displayed greater bias towards use of A and T than the other 2 clades, but the difference between *Rotaria* and *Brachionus* was not significant (Tukey's HSD test, $p > 0.5$). All other pairs of clades were significantly different. In 2 of the clades, *Adineta* and *Rotaria*, there was no significant difference in the frequency of G or C at silent third positions among clusters within the clade (anova, $F = 0.03$, $p > 0.5$ and $F = 0.2$, $p > 0.5$, respectively). The remaining 3 clades did exhibit significant variation in base composition among clusters, being strongest in *P. flaviceps* ($F = 82.3$, $p < 0.001$) and *Brachionus* ($F = 10.9$, $p = 0.002$) and weakest in *Daphnia* ($F = 5.6$, $p = 0.02$).

Discussion

Our findings provide the first evidence for inefficient natural selection in bdelloid rotifers. Across all 5 study clades, the extremely low average Ka/Ks ratios support a dominant pattern of purifying selection acting on the *cox1* gene. However, average Ka/Ks ratios were consistently higher in bdelloids than in the other 2 clades, as predicted if bdelloids have less efficient natural selection against deleterious amino acid mutations. The most likely reason why our results differ from previous studies comparing bdelloids and monogononts is that our sample included more recent relatives: the average Ka/Ks ratio derives from a larger sample of within-species or population variation than in previous studies. As well as increasing sample size, this type of sample is dominated by a different class of mutations, namely those that persist within populations but are removed over longer periods by selection.

This conclusion was confirmed by estimating separate Ka/Ks ratios within and between genetic clusters. Imple-

mentation of the clustering model identified significant clusters of closely related individuals in all 5 of the study clades, which we treated as population units for subsequent analyses. Optimizing Ka/Ks ratios separately within and between clusters revealed that the greatest variation among clades occurs for Ka/Ks within clusters. The Ka/Ks ratio on between-cluster branches does not vary consistently between bdelloids and the other clades. For example *P. flaviceps* has a between-cluster Ka/Ks ratio that is not significantly different from that of *Brachionus*, but its within-cluster Ka/Ks ratio is nearly 25 times greater (Fig. 3, Table 2).

We calculate the frequency of mildly deleterious amino acid mutations that persist as polymorphisms within clusters but are removed by selection over the longer-term as the difference between the within- and between-cluster Ka/Ks ratios (Paland and Lynch 2006a). In the bdelloid clades, between 3.2% and 9.7% of amino acid mutations fall into this class, whereas only 0.3% and 1.6% fall into this class in *B. plicatilis* and *Daphnia* respectively. Therefore, the results support the prediction that the purifying selection against mildly deleterious mutations is less efficient in the bdelloids than in the facultatively sexual clades. The lack of a systematic difference in amino acid fixation rate between clusters, assumed to represent nearly neutral mutations, could result from the lower frequency of this class of mutations or from non-zero rates of adaptive substitutions that vary among the clades (Weinreich and Rand 2004).

A number of assumptions influence interpreting these findings in the light of differences in sexuality among the study clades. Not least, the monophyly and age of bdelloid rotifers mean that repeated comparisons between closely related sexual and asexual lineages are not possible. Monogononts are the closest free-living relatives to bdelloids for which suitable population data are available, as yet only in sufficient number for the *B. plicatilis* complex. Yet, bdelloids and monogononts are likely to have diverged several hundred million years ago. *Daphnia* is even more distantly related, falling in a different major part of the metazoan tree (Ecdysozoa compared to Lophotrochozoa). Therefore, many other differences might explain the different patterns of DNA variation observed between bdelloids and the other clades. We discuss several possibilities in turn.

Population Size

The fixation probability of a new mutation depends on the product of the selection coefficient and the effective population size. Census population size is likely to be large in all the study groups based on the typical broad geographic distributions of species and genetic clusters (Dumont 1983; Adamowicz et al. 2004). If anything, bdelloids might have the largest census population sizes because of the ability of adults to survive desiccation and disperse passively (Jenkins and Underwood 1998; Cáceres and Soluk 2002). However, the effective population size also depends on the frequency of selective sweeps affecting the *cox1* gene. Because the entire bdelloid genome is strictly linked, this should be higher than in the other 2

clades. In fact, the estimate of theta, equivalent to the effective population size multiplied by mutation rate, indicates very little variation among the 5 study clades, and no consistent difference between bdelloids and the others. If effective population sizes were significantly smaller in bdelloids, which is the direction predicted to generate an increase in Ka/Ks ratios assuming all other things are equal, then their mutation rates would have to be higher to lead to no overall difference in theta. Relative rate tests of silent substitution rates between bdelloids and monogononts provide no evidence of different mutation rates affecting the *cox1* gene (Birky et al. 2005).

Codon Bias

Our analyses assume that silent changes are effectively neutral. If, instead, there were selection acting on silent sites, for example favoring usage of A and T over C and G, then this could change interpretation: the observed excess of Ka/Ks within clusters could result from a deficit of silent changes within clusters rather than an excess of amino acid changes. We did observe a systematic difference in base composition between the bdelloids and the other clades, with bdelloids having a higher frequency of A and T, although not significantly different in the comparison between *Rotaria* and *Brachionus*. However, stronger selection for base composition alone would tend to reduce the difference between the within- and between-cluster estimates of Ka/Ks ratio, not increase it, because silent changes would then be behaving more similarly to amino acid changes. To explain our results, there would have to be stronger *divergent selection* on base composition *between clusters* in the bdelloids than in the other 2 clades. However, if anything there is less variation among clusters in the bdelloids than in the other clades, since in *Adineta* and *Rotaria* we could not reject a null model of uniform base composition among clusters. Codon bias does not appear to explain the Ka/Ks ratio differences between bdelloids and the other clades.

Distribution of Mutational Effects

The analyses assume that the distribution of mutational effects is similar across the different study clades (Eyre-Walker et al. 2006). An alternative explanation for the high frequency of amino acid polymorphism within bdelloid clusters would be if the strength of selective constraint on *cox1* were less than in the other clades. In other words, relatively more of the mutations arising in bdelloids might have relatively low but non-zero selective coefficients. This seems impossible to distinguish from the effects of recombination based on *cox1* sequence data alone, since it depends on exactly what selection pressures the gene is under in each clade. Certainly, bdelloids living in terrestrial habitats will frequently experience higher temperatures than the other clades and higher temperature fluctuations, but one might expect this to create more stringent selection on the gene, if anything. Lacking alternative evidence, it could be argued that the recombination explanation is more parsimonious. All available evidence supports the asexuality of bdelloids, whereas we have no specific grounds to

suppose why the distribution of mutation effects at *cox1* should vary. Moreover, the recombination explanation relies on a generic effect whereas the mutational effects explanation requires specific differences affecting *cox1*.

Adaptive Polymorphism

The analyses assume that amino acid mutations are deleterious rather than beneficial, i.e., adaptive. An alternative explanation for the result is that bdelloid populations harbor adaptive polymorphism in *cox1* sequences. A putative case of adaptive polymorphism in *cox1* has been proposed in humans, perhaps reflecting local adaptation to diet or climate (Mishmar et al. 2003; Kivisild et al. 2006). The lack of recombination in bdelloids would make it easier to evolve adaptive polymorphism across suites of genes (Barraclough et al. 2003; Barraclough and Herniou 2003) and frequency-dependent coexistence of alternative genotypes has been proposed as a response to variable environments in other asexual species (Lynch 1987; Niklasson et al. 2004). However, frequency-dependent selection is expected to generate balanced polymorphisms, but the genealogical tests for history of selection supported a general deficit of mutations on more internal branches within clusters, the opposite pattern to that expected for balanced polymorphism. Also, once again, this explanation seems less parsimonious than the recombination explanation, because it relies on a specific form of selection acting on the *cox1* gene rather than general effects.

Population Expansion or Selective Sweeps

Independently of average effective population size, it has been shown that episodes of population expansion can bias McDonald-Kreitman tests comparing Ka/Ks ratios within and between populations (McDonald and Kreitman 1991; Eyre-Walker 2002). If recent population size is larger than past population size, then slightly deleterious mutations that do not segregate in the present may have become fixed in the past, when selection against them was less efficient. Recent selective sweeps affecting mtDNA would have qualitatively similar effects. However, this phenomenon seems unlikely to explain differences observed here. First, the finding of higher Ka/Ks ratios in bdelloids than in the other two clades would require that contemporary population sizes were smaller in bdelloids, irrespective of whether the sexual clades had experienced expanding populations or not. The estimates of theta do not support this assumption. Second, we found no consistent difference between asexual and sexual clades in population genetic statistics that test for population expansion or selective sweeps. There was a tendency towards negative values of both F_S and R_2 , the most powerful measures of population expansion or selective sweeps, but no consistent difference between the bdelloids and the other clades.

To summarize, although alternative explanations for the differences in Ka/Ks ratios between bdelloids and the other clades cannot be ruled out, the most obvious alternatives do not seem to explain the observed pattern. Interestingly, the population genetic measures of selection history, based on the shape of genealogy within

clusters, did not reveal consistent differences in the history of selection. While we might expect a strong pattern of purifying selection (low F^*) and higher frequency of selective sweeps (F_S and R_2) affecting *cox1* in the bdelloid clades, these tests are well known to be affected by several factors (Nielsen 2005), including changes in population size as discussed above. The advantage of the McDonald-Kreitman test in detecting selection on protein-coding sequences is its robustness to most confounding factors affecting the more general analyses of genealogical tree shape. Finally, sample sizes of individuals and of numbers of individuals within clusters did vary somewhat among the study clades, but we do not believe that this contributed to observed patterns. Although sample sizes within clusters were fairly low in all clades except *P. flaviceps* (table 1), our measures were obtained from averages across several clusters in each clade, yielding low standard errors for our estimates of K_a/K_s .

Conclusions

Our analyses provide the first evidence for inefficient selection in bdelloid rotifers, a classic clade of obligate and ancient asexuals. If confirmed by further studies on additional loci and sampling additional related sexual clades, for example more monogonont clades, the results will indicate that bdelloid populations do suffer the predicted effects of asexuality in terms of a high load of deleterious amino acid mutations. Despite this, bdelloids have survived and prospered over long evolutionary timescales. One possible explanation is that the impact of such mutations on fitness and population survival is less than has been supposed.

Supplementary Material

The supplementary table and Supplementary figures 1–5 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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