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'Ancient' DNA in the resting egg bank of a microcrustacean can serve as a palaeolimnological database

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Recent work on the diapausing egg banks of zooplankton, such as *Daphnia* (Crustacea: Anomopoda), indicates that these eggs can remain viable for decades while, theoretically, DNA can remain intact for even longer periods (i.e. centuries or millennia). We isolated diapausing eggs of *Daphnia* from a 30 m long sediment core taken from a hypereutrophic, northern German lake (Belauer See), with some eggs found in dated core material as old as 4500 years. Using microsatellite markers, we analysed the genetic structure of the resting eggs dated as old as *ca*. 200 years, and found that, although levels of heterozygosity remained remarkably stable, significant genetic differentiation (Nei's $D = 0.36$; $F_{ST} = 0.15$) between recent and 'ancient' resting eggs (including allele frequency shifts and private alleles) was detected. These shifts represent either species-level changes in this complex (i.e. species-specific characters of ephippia are not always robust), or intraspecific shifts in genetic variation, or a combination of both. This study demonstrates that the egg banks of aquatic zooplankton can serve as repositories of both genetic (intrapopulational) and ecological (interspecific) information. The use of molecular markers, such as microsatellites, on diapausing egg/seed banks may open new avenues of enquiry related to tracking the long-term genetic (and/or species) shifts that are associated with long-term environmental changes.

Keywords: egg banks; palaeolimnology; polymerase chain reaction; DNA; microsatellites

1. INTRODUCTION

With the advent of the polymerase chain reaction (PCR) (Saiki *et al*. 1988), the study of ancient biomolecules (i.e. DNA) has emerged as a major new field of enquiry (Pääbo *et al*. 1989). The results of such analyses have led to some spectacular claims of recovery and analysis of DNA from a 140-year-old quagga (Higuchi *et al*. 1984), a 330-year-old moa (Cooper *et al*. 1992), a 30-Myr-old termite (DeSalle *et al*. 1993), and a 120–135-Myr-old weevil (Cano *et al*. 1993). However, the authenticity of ancient DNA has often been called into question, because there is a high risk of contamination by extant DNA, as well as the lack of reproducibility in independent laboratories. But Krings *et al*. (1997) analysed Neanderthal DNA, which is assumed to be authentic because, recently, DNA sequence information has been obtained from a second Neanderthal specimen (Ovchinnikov *et al*. 2000), with the two specimens showing similar, but not identical, sequences. These studies indicate that, under certain environmental conditions, DNA molecules may resist post-mortem modifications (i.e. endogenous hydrolytic processes, oxidation or radiation; Pääbo *et al*. 1989) for substantial periods of time. Dark, anaerobic and cold lacustrine sediments should offer suitable conditions that would enhance the survival of DNA molecules for long periods of time (Höss 2000).

The microcrustacean zooplankter *Daphnia* reproduces via cyclical parthenogenesis, whereby direct-developing (subitaneous) eggs are produced apomictically during most of the year. Certain environmental cues, such as food limitation, high population densities, chemical and/or physical factors (Banta & Brown 1929; Hobæk & Larsson 1990) may initiate the switch to sexual reproduction and the production of diapausing (resting) eggs. Normally, one or two fertilized resting eggs are extruded into a sclerotized portion of the maternal carapace (termed an ephippium), which is shed upon moulting. A few *Daphnia* species are able to produce resting eggs via apomixis (Hebert 1981). Resting eggs enter diapause at an early gastrula stage (*ca*. 250 cells; Vollmer 1912) and need a stimulus for further development.

In intermittent or seasonally temporary waters, *Daphnia* depend on resting eggs for survival and dispersal between populations. By contrast, in permanent water bodies, such as lakes, many *Daphnia* populations are able to persist in the water column throughout the year, although this is not always the case (Wolf & Carvalho 1989). Therefore, resting eggs are not absolutely essential to guarantee population survival in some permanent water bodies, at least over the short term. However, in large, permanent lakes, an enormous number of resting eggs are produced (Wolf & Carvalho 1989; Weider *et al*. 1997). These resting eggs either float to the water surface or sink into the sediments, where they may accumulate over decades, centuries or millennia. Presumably, only a portion of the resting eggs will hatch from the egg bank each year, as a 'bet hedging' strategy (Cohen 1966; Hairston *et al*. 1996), and is akin to the well-known seed banks of terrestrial plants (Brown & Venable 1986). Previous studies (Hairston *et al*. 1995; Weider *et al*. 1997; Kerfoot *et al*. 1999) have recorded the chronological accumulation of the resting eggs of zooplankton in the annual sediment layers, which has permitted an accurate dating of sediments and eggs.

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Molecular techniques allow one to infer the genetic structure of an egg bank by directly examining the DNA held within dormant eggs (Duffy *et al.* 2000; Cousyn *et al.* 2001), thus precluding the need to induce eggs to hatch (Weider *et al.* 1997). Therefore, we isolated the resting eggs of *Daphnia* from a 30 m long sediment core taken from the varved/stratified sediments of the Belauer See (northern Germany), and sensitive microsatellite markers were applied to analyse the genetic structure of these eggs. Thus, a new dimension to the study of long-term temporal changes in both population and community structure is made accessible by using molecular methodologies to tap into the sequestered DNA of long-dormant propagules, such as diapausing eggs. We illustrate long-term genetic changes during the past two centuries, assessed via ancient and recent resting eggs of *Daphnia*.

The goal of this study is to demonstrate that molecular genetic methods (e.g. microsatellites) can be used to track long-term (century-long) shifts in the genetic structure of diapausing egg banks, with the potential to relate such shifts to the concomitant long-term environmental changes that influence both population and community structure in lake ecosystems.

2. MATERIAL AND METHODS

(a) *Study system*

The Belauer See is a relatively small (1.13 km^2), shallow (maximum depth 29 m, mean depth 9 m) lake located 30 km south of Kiel (Schleswig-Holstein), Germany. It is hypereutrophic with a sedimentation rate of *ca.* 2 ± 0.2 cm yr^{-1} (Garbe-Schönberg *et al.* 1998). *Daphnia* of the *longispina* species complex (i.e. *D. galeata*, *D. hyalina* and *D. cucullata*) are known to coexist in the lake (Wolf & Mort 1986).

(b) *Field collection, sediment core dating and processing*

In 1990, three 30 m sediment cores were taken at the centre of the Belauer See (> 28 m deep) and stored in a dark, cool room (< 4 °C) at the University of Kiel, Germany. The sediment core was dated to a depth of 23 m (7130 BC) by several methods, including counting stratified layers (Merkt 1994), stable isotope analysis, radiometric dating of elm tree falls (Erlenkeuser 1994) and pollen analysis (Wiethold 1998). Changes in the trophic state and pH history of the lake have been chronicled for the past 9000 years (Garbe-Schönberg *et al.* 1998).

In December 1998 and March 1999, surficial sediments were collected several times from the centre of the Belauer See (> 28 m deep) using an Ekman (Wildeo, Saginaw, MI, USA) dredge. The sediment samples were sliced with metal plates into an upper layer of *ca.* 2 cm thickness, which was taken as representative of the resting egg production in 1998, and three 4 cm thick lower layers, each representing *ca.* 2 years of sedimentation history.

From the 1990 sediment cores, 1.5 cm wide, 1–6 cm deep (equivalent to 2–20 years of sedimentation), and 1 cm thick sediment blocks were cut along the core to the extent that sediment was left from previous projects. From the 1998–1999 sediment layers, we used a plastic ring to 'punch out' subsamples (*ca.* 45.6 cm^3). To avoid possible inadvertent transfer of ephippia between different layers, these subsamples were taken from the middle of each layer. Sediment samples were placed in sealed 100 ml glass jars and stored at 4 °C in the dark. The remaining sediments were filtered through a metal sieve (250 μm mesh); ephippia were collected, and resting eggs were removed, counted and isolated for further DNA analysis. Ancient resting eggs that were isolated from the 1990 core, were processed in a completely separate, dedicated 'ancient DNA room', using sterile instruments, reagents and solutions that had not been in contact with extant *Daphnia* DNA.

(c) *Genetic analyses*

DNA was extracted from resting eggs by soaking them in 20 μl of 5% CHELEX 100 (Bio-Rad, Hercules, CA, USA) solution according to the protocol of Walsh *et al.* (1991). In certain instances, especially for the ancient resting eggs, we extracted DNA using the Forensic Kit I (Invitek, Germany) following a slightly altered manufacturer's protocol. Resting eggs were transferred into 250 μl of a solution containing a lysis buffer and 3.5 μl of a carrier suspension were added. The solution was vortexed, tubes were incubated for 5 min at room temperature and centrifuged at 10 000 rpm (touch-spin) for 1 s. Washing buffer was added (250 μl), mixed, centrifuged and the supernatant was decanted off. The last step was repeated twice. The sample was incubated for 20 min at 60 °C and the carrier pellet was resuspended in 20 μl elution buffer. Samples were stored overnight and then 1–1.5 μl was used for PCR amplification in a total volume of 10 μl. Four pairs of microsatellite primers designed by Dr John K. Colbourne (Indiana University, USA), and labelled Dpu 6 (GenBank AY057864) (Limburg 2000), Dpu 30 (GenBank AY057865) (Limburg 2000), Dpu 45 (GenBank AF23361) and Dpu 122 (AF23363), were used to amplify *ca.* 100–150 bp fragments, including dinucleotide repeats, with the exception of Dpu 122, which amplifies a trinucleotide repeat. Forward primers were end-labelled with fluorescent dyes (PE Biosystems, Foster City, CA, USA). PCR reactions were carried out with final concentrations of 0.3 μM of each primer, 0.5 μM of dNTP, 1–2 mM of $MgCl_2$, either 2.5% DMSO or 5% DTT, and 0.25 units per reaction of *Taq* DNA polymerase (Promega, Madison, WI, USA). PCR amplifications commenced with denaturation for 2 min at 95 °C, followed by 35 cycles of: 95 °C denaturation for 30 s; annealing at 43–56 °C for 30 s; 72 °C extension for 1 min, followed by a final 72 °C extension for 20 min. Microsatellite alleles were amplified, resolved on a denaturing polyacrylamide gel and visualized using an automated DNA sequencer (PE Biosystems; ABI 377).

(d) *Statistical analyses*

To measure genetic variation and allelic diversity among ancient and recent resting eggs, we used the Tools For Population Genetic Analyses (TFPGA) program of Miller (1997) to examine allele frequency differences, unbiased estimates of heterozygosity and gene diversity (expected under the Hardy–Weinberg equilibrium). We further used the ARLEQUIN program (Schneider *et al.* 2000) to examine deviations from Hardy–Weinberg expected genotype frequencies, as well as linkage disequilibria among paired loci. The population structure of recent and ancient resting eggs was estimated on the basis of the variances in allele sizes using F_{ST} (Wright 1921; Weir & Cockerham 1984), as a measure of population genetic differentiation.

3. RESULTS

Figure 1 shows examples of ancient ephippia isolated from the sediments of the Belauer See. The resting eggs

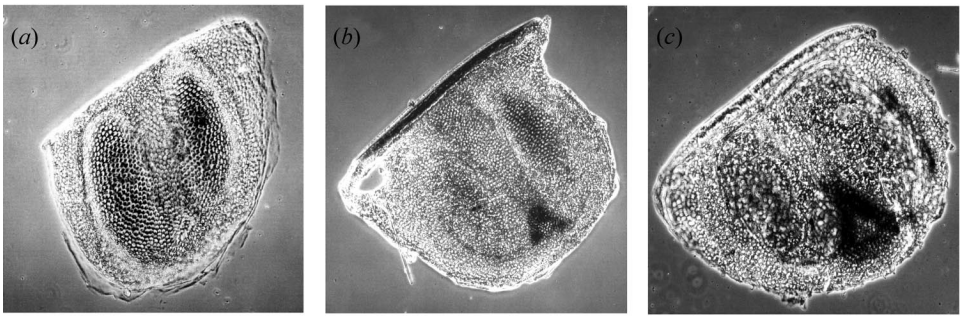

Figure 1. Ancient ephippia from the Belauer See sediments. (*a*) 550-year-old *Daphnia galeata*; (*b*) 1750-year-old *Daphnia pulex* and (*c*) 3300-year-old *Daphnia cucullata* ephippiua.

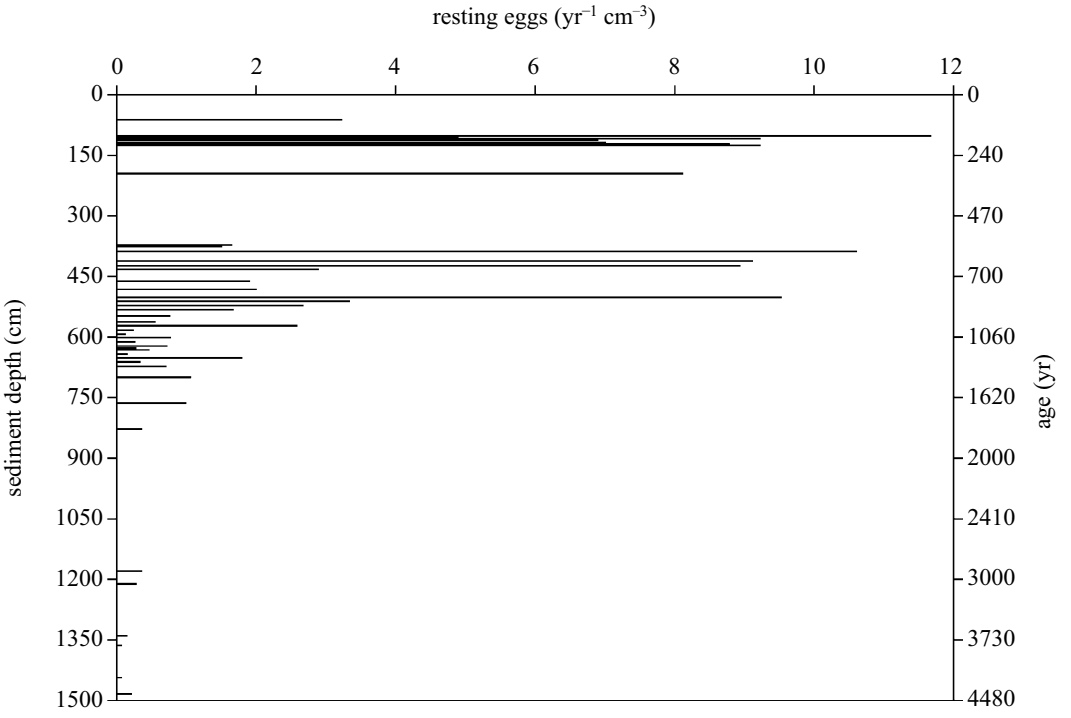

Figure 2. Abundance of ancient resting eggs in the Belauer See sediments.

within the ephippial casings were mostly dehydrated. One can see that the external degradation of the ephippial casing seems to be relatively similar among the differently aged ephippia, which ranged in age from 550 years old (figure 1*a*, *D. galeata*) to 1750 years old (figure 1*b*, *D. pulex*, a species no longer found in the extant fauna) to an astounding 3300-year-old ephippium (figure 1*c*, *D. cucullata*). Although it was possible to distinguish individual *D. pulex* ephippia (due to their specific shape) from members of the *D. longispina* group (*D. galeata*, *D. hyalina* and *D. cucullata*), it was often difficult to distinguish between members of the *D. longispina* complex, particularly between *D. galeata* and *D. hyalina*, because of damage to ephippial casings. Therefore, our microsatellite analyses were conducted on mixed samples of ephippia from this complex and, therefore, represent both intra- and interspecific shifts in genetic variation in the egg bank.

Resting egg densities (figure 2) were highly variable in the sediments of the Belauer See. Because the 1990 core had been intensively sampled during previous projects, it was not possible to analyse and compare replicate subsamples from the same depth. The highest densities of resting eggs were found in the upper 6 m of the core, i.e. the past *ca*. 1000 years. These estimates are based on 1040 resting eggs isolated from recent (0–14 cm) and 2400 resting eggs isolated from ancient sediments (62 cm to 15 m).

These densities are several-fold higher than estimates obtained from the surficial sediments collected in 1998 and 1999. In general, resting egg abundances were highly variable until *ca*. 1000 years ago, when they suddenly showed a dramatic decrease in abundance with increasing depth (age). For the oldest sediments (4400 years old) analysed in this study, abundances of 0.2 resting eggs yr^{-1} cm^{-2} were calculated.

Genetic analysis of ancient resting eggs was performed on very small amounts of (possibly degraded) DNA. Hence, the PCR success of ancient microsatellite sequences was considerably reduced, when compared with results obtained from recent resting eggs. Nevertheless, we did succeed in amplifying microsatellite alleles from more than 100 ancient resting eggs. To confirm our results, we carried out replicate PCR reactions for each microsatellite locus and for each resting egg. Every resting egg was

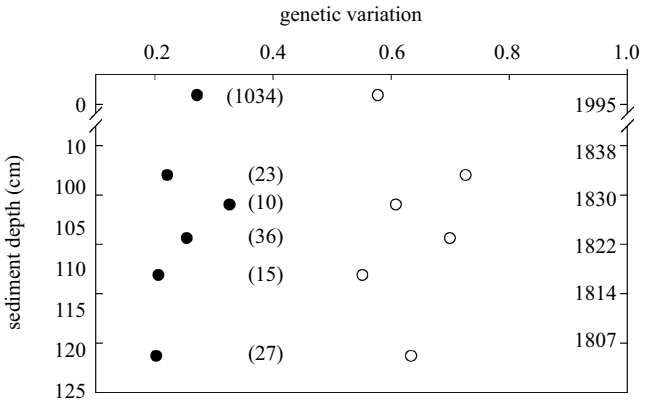

Figure 3. Genetic variation of recent (0–7 years) and ancient (150–195 years) resting eggs of the *Daphnia longispina* group isolated from a 30 m sediment core. Filled circles denote heterozygosity, open circles denote gene diversity. Sample sizes are in parentheses.

derived from a different ephippium. The analysed ancient resting eggs were isolated from sections of core taken from a depth range of 1–1.30 m, which corresponds to an age range of *ca*. 159–195 years (figure 3; Wiethold 1998).

Heterozygosity and gene diversity estimates for recent and ancient resting eggs (figure 3) were comparable, with estimates for heterozygosity ranging from 0.20 to 0.32, and gene diversity estimates ranging from 0.55 to 0.73.

A comparison of microsatellite allele frequencies for the four loci from ancient and recent resting eggs is given (figure 4). Unfortunately, damaged ephippial casings prevented an unambiguous determination of *Daphnia* taxa. Thus, allele frequencies depicted in figure 4 represent the pooling of all *Daphnia* taxa from the *longispina* complex that co-occurred in the Belauer See. Allelic richness was higher for ancient resting eggs (average ± 1 s.e. of 8 ± 1.5 alleles for the four loci), compared with recent resting eggs (average 6.2 ± 1.9), although not significantly so. Several private or unique alleles were detected among the ancient resting eggs examined (e.g. allele 145 at Dpu 30; allele 133 at Dpu 45; figure 4), but were not found in either the recent resting egg bank or the live population of *Daphnia* in the water column (data not shown). Although *Daphnia* in the Belauer See water column and the recent resting egg bank differ in allele frequencies, both show nearly identical microsatellite allelic arrays. Further, several private alleles were detected in the recent egg bank (e.g. allele 155 at locus Dpu 30; allele 127 at locus Dpu 45; figure 4), but were not found in the ancient resting egg bank. The greatest number of alleles for both the recent and ancient resting eggs was found at locus Dpu 6 (figure 4). Only one out of 111 multi-locus composite genotypes was common between the recent and ancient egg banks.

Allele frequencies between recent and ancient resting eggs were significantly different from each other ($p < 0.0001$), which resulted in a large genetic distance estimate ($D = 0.36$; Nei 1972). Furthermore, the F_{ST} value ($F_{ST} = 0.15 \pm 0.04$) indicates considerable genetic differentiation between recent and ancient resting eggs.

4. DISCUSSION

In contrast to other studies, which have dealt with single discoveries of ancient DNA (e.g. a single, 17-Myr-old magnolia (Golenberg *et al.* 1990)) or individual human findings (i.e. Hänni *et al.* 1994; Handt *et al.* 1996; Krings *et al.* 1997; Haas *et al.* 2000; Ovchinnikov *et al.* 2000), in the Belauer See, an enormous number of *Daphnia* resting eggs is preserved chronologically in the sediments. Stratified layers enable one to accurately date sediments and resting eggs and, therefore, allow one to study *Daphnia* population genetic structure across long-term time periods. Allozyme analysis permits one to investigate live hatchlings of *Daphnia* (Weider *et al.* 1997), but not resting eggs (with the lone exception of the *Pgi* locus, L. J. Weider, personal observation). Accordingly, allozyme analysis is somewhat restricted to the analysis of viable resting eggs, which can be induced to hatch. Though *ca*. 330-year-old resting eggs of copepods were induced to hatch (Hairston *et al.* 1995), successful hatching of most *Daphnia* resting eggs is restricted to *ca*. 60–70 years, or less (Weider *et al.* 1997; Cáceres 1998; Hairston *et al.* 1999*a*). However, the use of microsatellites permits one to analyse non-viable resting eggs, which often contain minute amounts of non-degraded/unmodified DNA, as demonstrated in our study, and also in a recent paper by Cousyn *et al.* (2001).

Although not all sediment depths from the 30 m core were available for analysis, and therefore a systematic analysis through a substantial period of time was not possible, we were still able to examine several interesting aspects.

The abundance of ancient resting eggs fluctuates to a great extent among different sediment depths (figure 2). According to the results of diatom analyses of this same core (Garbe-Schönberg *et al.* 1998; H. Håkansson, personal communication), the Belauer See was categorized as being mesotrophic, at least up until 2000 years ago. Subsequently, during the past 2000 years, indicator diatoms such as *Stephanodiscus minutulus* and other alkaliphilous forms became more prevalent, thus indicating increased eutrophication. Previously predominant *Cyclotella* sp., which is known to prefer oligo- to mesotrophic water bodies, disappeared about 1060 years ago (i.e. 6 m core depth). During the past several decades, the Belauer See has been reclassified as hypereutrophic. Although *S. minutulus* dominated during most of the past 2000 years, it is rare today.

If the changes in trophic state of the Belauer See (Garbe-Schönberg *et al.* 1998) are compared with changes in resting egg abundances during this same time period, some interesting patterns emerge. In particular, fairly high resting egg abundances were detected dating back *ca*. 900 years, thus, shortly after *Cyclotella* sp. disappeared. Fluctuations in resting egg abundances are pronounced, so that a sampling effect (i.e. a patchy distribution of resting eggs in sediments or limited sample size) cannot be discounted. Perhaps, fluctuating abundances reflect temporal changes in *Daphnia* population densities. However, the time-scale of the diatom analysis, as well as of resting eggs, exhibits several gaps. Thus, a relationship between changes in environmental conditions (i.e. lake trophic state changes) and changes in resting egg abun-

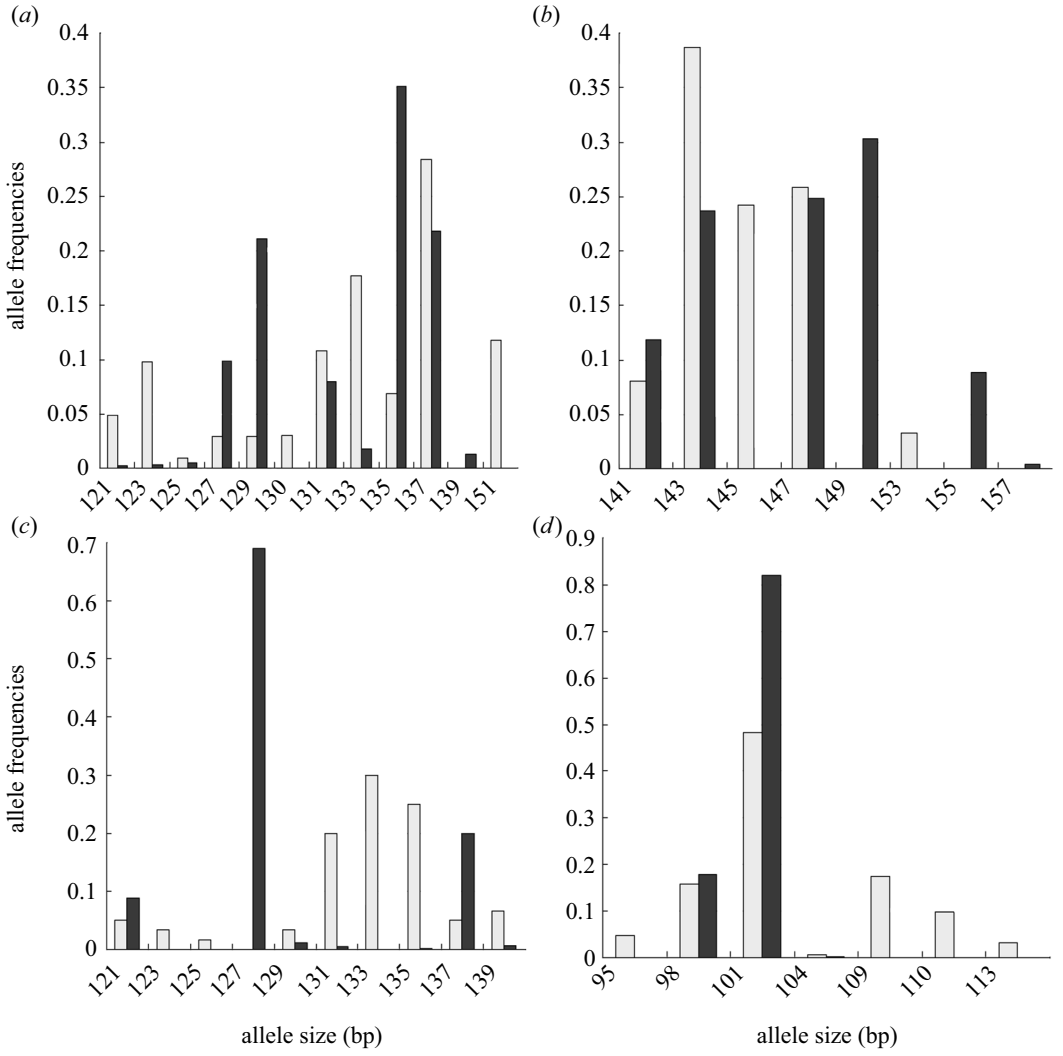

Figure 4. Allele frequencies of microsatellite loci (*a*) Dpu 6; (*b*) Dpu 30; (*c*) Dpu 45 and (*d*) Dpu 122 for recent (0–7 years; sample size $n = 848$; black bars) and ancient (150–195 years; $n = 111$; grey bars) resting eggs of the *Daphnia longispina* group. bp, base pairs.

dances from distinct time periods (i.e. distinct decades or centuries) remains somewhat problematic.

A comparison of the genetic variation (figure 3) obtained from the ancient (159–195-year-old) egg bank, indicates no significant differences when compared with the recent (0–7-year-old) egg bank. Nevertheless, it does appear that considerable genetic changes have occurred during the past 200 years, as exemplified by significant genetic distance ($D = 0.36$) and substantial genetic differentiation ($F_{ST} = 0.15$) between recent and ancient resting egg banks. Furthermore, detailed analysis might reveal even more genetic differences. However, these observed differences indicate that microevolutionary processes have had a role here, influencing either intra- or interspecific variation. This supposition is further supported by the observation that several private (unique) alleles were only detected among the ancient resting eggs, whereas significant differences in microsatellite allele frequencies were detected between recent and ancient egg banks (figure 4). Although we cannot exclude the possibility that some alleles may have been missed due to sampling error, it is clear that private alleles are definitely present (e.g. locus Dpu 122, figure 4).

Previous studies (Weider *et al.* 1997; Hairston *et al.* 1999*a*; Cousyn *et al.* 2001) have demonstrated that microevolution can occur in populations of well-defined *Daphnia* resting egg banks, which date back over a period of *ca.* 30–40 years. By using microsatellites, as in this present study, we can therefore attempt to extend this time-scale back to almost 200 years ago (or longer). Furthermore, molecular markers can help us look not only at intrapopulational changes, but also at community or assemblage-level changes. For example, resting eggs of *D. pulex* were found in the Belauer See sediments spanning several centuries (as well as a 1750-year-old *D. pulex* resting egg as shown in figure 1*b*). *D. pulex* is no longer present in the extant zooplankton fauna of the Belauer See. Environmental changes in a water body may influence invasion and extinction of *Daphnia* species (Hairston *et al.* 1999*b*; Duffy *et al.* 2000; Reid *et al.* 2000). If one assumes that these resting eggs of *D. pulex* did not drift into the sediments from outside the basin, but reflect autochthonous production in the water column of the Belauer See in the past, this may serve as a tool for examining the effect of changing environments not only on populations, but also on communities.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.