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Isolation, amplification, and identification of ancient copepod DNA from lake sediments

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Abstract

Species identification of copepods in lake sediments is often difficult because their remains lack diagnostic features. It is therefore not easy to track changes in copepod biodiversity in lakes through time. We report a method for the isolation, amplification, and identification of copepod DNA from whole lake sediments formed in the early Holocene to the present. The method, which involves amplification of a short (~300 base pair) DNA sequence that varies between copepod species, provides a new approach to the study of copepod paleobiodiversity. Successful amplification of copepod DNA was possible in samples as old as 9950 calibrated ^{14}C y BP. Attempts failed to recover DNA from a sediment sample ca 65,000 years old. In most cases the species identified in the sediments matched those of extant lake populations, but analysis of early-mid Holocene sediments from one lake revealed a species that is not present today. We were able to recover copepod DNA from core samples stored at –20° C, at 4° C, and preserved with polyethyleneglycol.

Development of reliable protocols for the recovery of ancient DNA preserved in lake sediments is an experimental goal that is of major importance for studies of climate-induced changes in lacustrine biodiversity and biogeography. Knowledge of past distributions of organisms allows more critical analysis of present-day biogeography and deeper understanding of how these distributions have developed. Records of change found in lake sediments have been used widely in reconstructing past communities (Cohen 2003). However, these paleoecological studies are limited to taxa that leave easily identifiable remains, such as diatoms, cladocerans, and chironomids, (e.g., Frey 1964; Smol et al. 2001, 2005). The occurrence of these well-studied taxa veils the apparent absence from the sedimentary record of remains from many other species, and therefore the paleoecological and biodiversity information they could yield is missing. Development of reliable methods for identifying the presence of otherwise cryptic species in lakes at some time in the past would have significant applications to understanding how both the ecology of individual lakes and the present-day distributions of species have developed. One approach is to look for chemical evidence for the occurrence of a species; whereas use of chemical biomarkers is used widely (Meyers 1997; Meyers and Terranes 2001; Leavitt and Hodgson 2001), a more objective method is the analysis of DNA.

Isolation of DNA from lake sediments has been achieved previously by two approaches. Duffy et al. (2000), Cousyn et al. (2001), Limburg and Weider (2002), and Reid et al. (2000, 2002) reported the isolation and amplification of DNA from ephippial (resting) eggs of *Daphnia* sp. (Cladocera) picked from lake sediments. DNA was successfully recovered from samples up to 200 years old, although no limit to the age of ephippia from which DNA could be recovered was determined (Limburg and

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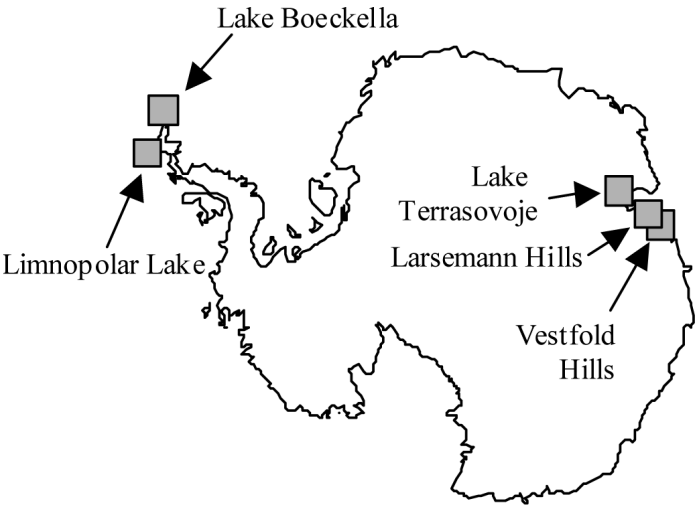

Fig. 1. Map of Antarctica showing the locations of sampling sites and other places mentioned in the text.

Weider 2002). Ephippial eggs can remain viable in the sediment for decades or centuries (Hairston 1996), and it is therefore not surprising that DNA could be isolated from this source. These studies have shown clearly the utility of molecular genetic methods to separate morphologically similar congeners in the investigation of changes in cladoceran populations (e.g., invasions of new species) in lakes (Duffy et al. 2000). However, this approach requires time-consuming isolation of the eggs from the sediment, and also provides no information about other, more cryptic species present. In particular, this approach has not been applied to copepods even though many species produce long-lived diapause eggs (Hairston 1996).

The second approach is to extract DNA from bulk sediment, which was first reported for the identification of purple sulfur bacteria in a Canadian lake (Coolen and Overmann 1998). More recently, the method was applied by Coolen et al. (2004a, b) to sediments from Ace Lake, Antarctica. These authors identified archaeal, bacterial, and algal DNA and were able to follow the development of the methane cycle within the lake. However, this approach appears to have been applied only to prokaryotic or unicellular eukaryotic groups.

Here we report the successful isolation and amplification of copepod DNA from bulk lake sediments. We identified copepods that lived in 3 Antarctic lakes up to 9950 calibrated ^{14}C y before present (BP) by sequencing a ~300 bp fragment of DNA amplified using copepod-specific primers. The general approach described here is applicable to other metazoan groups.

Materials and procedures

Sediment collection—Sediments from 4 fresh to slightly brackish Antarctic lakes were collected and stored as follows. Two sediment cores were recovered from Lake Terrasovoje, Northern Prince Charles Mountains (70° 33′ S, 68° 2′ E; see Fig. 1 for location of sampling sites and Wagner et al. [2004] for further details of this lake), in December 2003 using a UWITEC piston corer. One core was sectioned into 1- or 5-cm segments and placed into sterile Whirl-pak plastic bags in the field, and the other was frozen whole. The segments were stored at 4° C from collection until analysis, and the frozen core was maintained at –20° C. A 2.93-m-long core from Lake Boeckella, Hope Bay (63° 24′ S, 57° 0′ W), was collected in November 1987 (Zale and Karlén 1989). It was cut into 5-cm segments and stored at 4° C. The core was subsequently (within months) preserved by displacing the water in the sediment with polyethyleneglycol (PEG) (Tippet 1964) and stored at 4° C. A 29.2-cm-long core was obtained in December 2003 from Limnopolar Lake, Byers Peninsula, Livingston Island (62° 38′ S, 61° 4′ W), using a Glew gravity corer. This core was sectioned in the field and stored at 4° C. Finally, a core from Lake Reid, Larsemann Hills (69° 23′ S, 76° 23′ E), was obtained as described previously (Hodgson et al. 2001, 2005). This core was sectioned in the field and stored at 4° C until analysis.

Sediment sample handling and DNA extraction—Isolation of DNA was undertaken between July and December 2004, less than a year after collections of the cores from Lake Terrasovoje and Limnopolar Lake, 6 years after the collection of the core from Lake Reid, and 16 years after the collection of the core from Lake Boeckella that had been subsequently preserved with PEG.

The general laboratory protocol, designed to minimize the chance of contamination, was similar to that described previously for the isolation of DNA from lake sediments (Coolen and Overmann 1998). All sampling and amplification reactions were performed in a UV-sterilized laminar flow cabinet that was used exclusively for PCR work. All glassware and plastic-ware was UV sterilized and was replaced after each sediment horizon had been extracted. Only one sediment horizon was extracted at a time. Pipettes used for DNA extraction were not used for PCR, and pipette tips with sterile sealing filters were used to prevent contamination by aerosols. Disposable gloves were always worn and were changed at the completion of each step in the sampling procedure (e.g., removal of sediment, each step in the DNA extraction). Parallel blank samples were subjected to the extraction, purification, and PCR procedure as negative controls (sediment-free controls). Each PCR amplification series included one reaction without DNA template, which served as a further negative control (NTC). Extractions and amplifications with a second sediment sample ensured repeatability of results. Details of all samples extracted are given in Table 1.

Several precautions were taken against contamination of samples with foreign DNA. For sediment samples contained in Whirl-pak bags, the outside of the bag was initially washed with 95% ethanol and dried. Samples were taken from the center of the core plug within the bag using a sterile scalpel blade. For the frozen core, the depth horizon to be analyzed was cut from the core tube using a sterilized hacksaw, the frozen sediment was extruded, and the outer surfaces were cut away using sterile scalpel blades. Material for extraction of DNA was taken from the center of the sample with a fresh scalpel blade. When multiple horizons were sampled from the

Table 1. Details of samples extracted in this study.

Lake	Depth (cm)	Estimated age	Preservation method	*Boeckella poppei*	Unidentified Acartiid
Terrasovoje	0-1	Modern	4° C	AY997795	–
Terrasovoje	30-31	3430*	4° C	AY997794	AY997793
Terrasovoje	160-165	9950*	4° C	AY997791	AY997792
Terrasovoje	160-165	†	–20° C	+	+
Reid	23-24	3750‡	4° C	–	–
Reid	93-94	65,000‡	4° C	–	–
Limnopolar	0.2-0.4	<50	4° C	AY997811	
				AY997812	–
Limnopolar	28-29.2	2020*	4° C	AY997814	–
Boeckella	0-3	Modern	PEG/4° C	AY997813	–
Boeckella	288-293	5850§	PEG/4° C	AY997815	–

GenBank accession numbers are given where relevant.
+ indicates sequences identified in samples but not submitted to GenBank; –, no copepod sequences were recovered.
*Calibrated radiocarbon age – median probability from the CALIB Radiocarbon Calibration Program revision 5.0 (Stuiver and Reimer 1993) using the Southern Hemisphere calibration curve (McCormac et al. 2004). Full details of these radiocarbon age determinations are given in Table 2.
†The two cores from Lake Terrasovoje were collected within a few meters of each other. A radiocarbon age was not determined for the sample from 160-165 cm in the frozen core, but should be close to that at the same depth in the core stored at 4° C.
‡Hodgson et al. (2005).
§Modeled by Zale (1994).

same core in the same session, samples were isolated from the deepest (i.e., oldest) sediment first.

DNA was extracted from 0.5 g of sediment using soil DNA extraction kits (Bio101, Vista, CA, USA). Extractions largely followed the manufacturer's instructions, except that a second, additional extraction and cell disruption step using a Mikrodismembrator U bead beater (B. Braun Biotech International, Melsungen, Germany) was employed. The first comprised two 10-s periods at 3800 rpm, after which the supernatant was removed and replaced with 700 µL Na_2HPO_4. The sample was then beaten at 5000 rpm for 30 s. The supernatant from the second beating was pooled with the first, and the extraction procedure was completed according to the manufacturer's instructions. Total DNA extracts were subjected to agarose gel electrophoresis and ethidium bromide staining to determine DNA quality and fragment size. Final extractions (50 µL) were stored at –20° C.

DNA extraction from copepod samples—Total genomic DNA was extracted from ethanol-preserved copepod specimens collected from Antarctic lakes or nearby marine waters (Table 3). Copepods were soaked overnight in MilliQ water before extraction. One to several copepods were placed into a sterile 1.5-mL microcentrifuge tube and crushed with a pestle in liquid N_2. The samples were then processed using Qiagen DNEasy tissue extraction kits according to manufacturer's instructions. Total DNA extracts were subjected to agarose gel electrophoresis and ethidium bromide staining to determine DNA quality and fragment size. Final extractions (50 µL) were stored at –20° C.

Polymerase chain reaction—Partial fragments of the 18S and 28S rRNA genes were amplified using PCR. Amplifications were performed as 25-µL reactions using the Clontech Advantage 2 Polymerase System (BD Biosciences), according to manufacturer's instructions.

Table 2. Details of AMS ^{14}C age determinations for samples from Lake Terrasovoje and Limnopolar Lake.

Lake	Sample depth (cm)	Laboratory number	^{14}C age ± 1σ error	2σ calibrated age ranges	Relative area under distribution	Median age
Terrasovoje	30-31	OZH949	3260 ± 60	3270-3290	0.02	3430
				3320-3580	0.98	
Terrasovoje	160-165	OZH950	8920 ± 90	9630-9640	0.01	9950
				9660-10,210	0.99	
Limnopolar	28-29	OZH859	2110 ± 50	1890-2150	1.00	2020

Calibrated ages were calculated using the program CALIB (version 5) (Stuiver et al. 2005). The Southern Hemisphere calibration set (McCormac et al. 2004) was used for all samples. No reservoir correction was applied, and all ages have been rounded to the nearest 10 years.

Table 3. Copepod species sequenced using the CopF2 and CopR1 primers. All lakes and bays mentioned specifically, with the exception of Lake Terrasovoje, are in the Vestfold Hills, Antarctica. Samples from the Larsemann Hills were collected immediately offshore from China's Zhong Shan research station.

Species	Sampling site	Location	GenBank accession number
Acanthocyclops mirnyi	Watts Lake	68° 36.20′ S 78° 13.16′ E	AY997816
Amphiascoides sp.	Lake Abraxas	68° 29.33′ S 78° 17.22′ E	AY997801
Boeckella poppei	Beaver Lake	70° 47.39′ S 68° 14.33′ E	AY997810
Boeckella poppei	Lake Terrasovoje	70° 33.32′ S 68° 01.54′ E	AY997809
Calanus propinquus	Antarctic pack ice	64° 33.44′ S 116° 35.28′ E	AY997808
Drepanopus bispinosus	Snezhnyy Bay	68° 26.35′ S 78° 26.59′ E	AY997805
Drepanopus bispinosus	Taynaya Bay	68° 27.15′ S 78° 17.54′ E	AY997807
Oithona similis	Larsemann Hills	69° 22.30′ S 76° 22.60′ E	AY997802
Paralabidocera antarctica	Lake Abraxas	68° 29.33′ S 78° 17.22′ E	AY997800
Paralabidocera antarctica	Ace Lake	68° 28.31′ S 78° 11.27′ E	AY997798
Paralabidocera antarctica	Larsemann Hills	69° 22.30′ S 76° 22.60′ E	AY997803
Paralabidocera antarctica	Pendant Lake	68° 27.73′ S 78° 14.41′ E	AY997799
Paralabidocera antarctica	Taynaya Bay	68° 27.15′ S 78° 17.54′ E	AY997806
Stephos longipes	Larsemann Hills	69° 22.30′ S 76° 22.60′ E	AY997804

Initially the 18S small subunit (SSU) rRNA gene in extracted DNA was amplified using the bilaterian-specific primers BilSSU1100F and BilSSU1300R (Jarman et al. 2004) with the following thermocycler conditions: 94° C for 4 min; 30 cycles of 94° C for 10 s, 62° C for 30 s, and 72° C for 30 s; and a final 7-min extension step. This PCR was carried out to ensure that successful extraction of amplifiable DNA from the lake sediments was possible. Once we had confirmed the presence of arthropod DNA, copepod-specific primers were developed and applied to the same samples.

Primers for amplifying a portion of the D domain of the nuclear large subunit rDNA (Wuyts et al. 2001) from calanoid, cyclopoid, and harpacticoid copepods were developed. All sequences available in GenBank for these copepod groups and for non-copepod crustaceans were aligned using ClustalX (Thompson et al. 1997). Appropriate copepod-specific primers for nested PCR were designed with the software Amplicon (Jarman 2004). The primers were tested empirically for their ability to amplify copepod rDNA to the exclusion of rDNA from other crustacea, including ostracods, amphipods, krill, isopods, barnacles, and crabs, as well as gastropods, ctenophores, cnidaria, echinoderms, chaetognaths, and fish.

Sediment DNA samples and all control samples were subjected to nested PCR amplification of the 28S rRNA gene using primers F63 (GCATATCAATAAGCGGAGGAAAAG) and R635 (GGTCCGTGTTTCAAGACGG), followed by CopF2 (TGTGTGGTGGTAAACGGAG) and CopR1 (CCGCCGACCTACTCG). Thermocycler conditions for the F63-R635 PCR comprised an initial denaturation step of 4 min at 94° C; 18 cycles of 94° C for 30 s, 62° C for 45 s (decreasing by 0.5° C each cycle), and 72° C for 60 s; 10 cycles of 94° C for 30 s, 52° C for 30 s, and 72° C for 60 s; and a final 72° C extension step of 4 min. For the CopF2-CopR1 PCR, the following thermocycler conditions were employed: 94° C for 60 s; 29 cycles of 94° C for 5 s, 61° C for 20 s, and 72° C for 30 s; and a final 72° C extension step of 10 min. All PCR products were subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide to confirm the presence of DNA fragments of the correct length and to

ensure the absence of contamination in the NTC and sediment-free negative controls. Copepod samples were amplified in a single PCR run using primers CopF2 and CopR1 with the thermocycler conditions above.

Cloning and DNA sequencing—Seven sediment horizons were chosen for clone library construction: three from Lake Terrasovoje, two from Lake Boeckella, and two from Limnopolar Lake. Attempts to isolate DNA from both modern and ancient sediment from Lake Reid failed, and no clone library could be produced from these samples. Further details and estimated ages of sediment samples used in this study are given in Table 1. The TOPO cloning system was used to ligate PCR fragments from these horizons into the pCR-TOPO vector and transformed into Epicurian Coli ultracompetent cells (Invitrogen, Carlsbad, CA, USA). Transformants were screened using blue-white selection on Luria agar containing Xgal and 100 μg mL^{-1} ampicillin. White colonies were transferred to fresh plates and reincubated overnight. Plasmids were extracted using the Ultraclean miniplasmid extraction kit (Mo Bio, Carlsbad, CA, USA). Positive clones were sequenced with the Beckman Ready Reaction Dideoxy Cycle sequencing kit (Beckman Coulter Inc., Fullerton, CA, USA) and M13 forward and reverse primers, and sequencing was performed with the Beckman CEQ2000XL automated capillary sequencing system.

The chimera check tool of the Ribosomal RNA Database Project (http://rdp.cme.msu.edu; Cole et al. 2005) was used to check possible chimeric sequences. Sequences were aligned with reference sequences obtained from the National Center for Biotechnology Information (NCBI) nucleotide database (Altschul et al. 1997) using BioEdit (version 5.0.9) (Hall 1999). Sequence similarity trees were created by calculation of maximum-likelihood distances and by using the UPGMA algorithm through the BioEdit program. Trees were created from the Neighbor output by using the program Treeview (Page 1996). An orthologous DNA sequence from the crayfish *Cherax crassimanus* (AY211597) was used as the outgroup reference for all trees.

Nucleotide sequence accession numbers—Copepod sequences generated in this study have been deposited in GenBank under the accession numbers AY997791 to AY997816 (see Tables 1 and 3 for further details).

Microscopic identification of copepod remains—Searches were made for copepod remains in each of the core segments analyzed as follows: ~1 g wet sediment was placed into 10 mL distilled water along with a few drops of the stain Rose Bengal. The sediments were let stand overnight to disaggregate at 4° C and then passed through a stack of filters with mesh size 200 μm, 100 μm, and 44 μm. Samples were examined using a dissecting microscope, and copepod eggs and spermatophores were identified by comparison to modern material.

Radiocarbon dating—Radiocarbon-based ages were available for all sediments either through data in the literature or measurements made at the Australian National Science and Technology Organization using accelerator mass spectrometry (AMS) techniques. Details of the ages are given in Table 1.

Assessment

The copepod-specific primers CopF1 and CopR2 were tested successfully on 14 copepod samples collected from Antarctic marine and lacustrine environments. The genetic tree developed from these samples and the sequences available from GenBank was largely congruent with views of copepod evolution based on other evidence (Fig. 2) (Huys and Boxshall 1991). For example, the more derived families group together (e.g., Clausocalanidae and Stephidae), and the clade from Metridinidae to Paracalanidae is consistent with morphology-based views. Excellent separation was observed at a family level; separation at generic and specific levels, although good in some cases, was more equivocal.

DNA extraction from the lake sediments was successful for all samples except those from Lake Reid. Probable reasons for this failure are discussed below. The initial PCR amplification of the 18S rRNA gene using the BilSSU primers returned only sequences that grouped with arthropods, but the fragment lacked sufficient resolution to identify which arthropod group was represented (data not shown). Blank extractions failed to show the presence of DNA or PCR amplification products, except for occasional PCR NTC reactions with the BilSSU primers that showed amplification products. This occurred rarely (twice) and only with this non–copepod-specific primer set. When contamination was detected, the PCR was repeated using freshly made reagents. Uncontaminated PCR was always achieved in the subsequent reaction.

PCR amplification of sediment DNA with primers CopF1 and CopR2 was successful and indicated the presence of copepod DNA in all sediment samples studied except those from Lake Reid (Table 1). Clone libraries were successfully constructed from 8 sediment samples (Table 1). Two distinct DNA sequences were isolated from Lake Terrasovoje sediment. The first was recovered from all samples investigated and clustered closely with modern *Boeckella poppei* collected from both Lake Terrasovoje and nearby Beaver Lake (Bayly et al. 2003) (Fig. 2). The second sequence, which was found only in samples from 30-31 cm and 160-165 cm (stored at both 4° C and –20° C), grouped closely with *Paralabidocera antarctica*, a marine species of the family Acartiidae that is also found in some Antarctic lakes (Swadling et al. 2004). At first this occurrence was thought possibly to be contamination, as the presence of marine species in slightly brackish Lake Terrasovoje seemed anomalous. However, the absence of this sequence from the surface sediment and from the sediments of other lakes studied, careful attention to blanks, and its occurrence in two independent cores, has convinced us of its reliability (see below for further discussion on why we dismiss contamination as a source for either sequence). Furthermore, there was no indication from the results of this study that the species present was necessarily *Paralabidocera antarctica*. Analysis of further samples and longer sequences would be required to refine the relationship of this species to others in the family Acartiidae and confirm this possible identification.

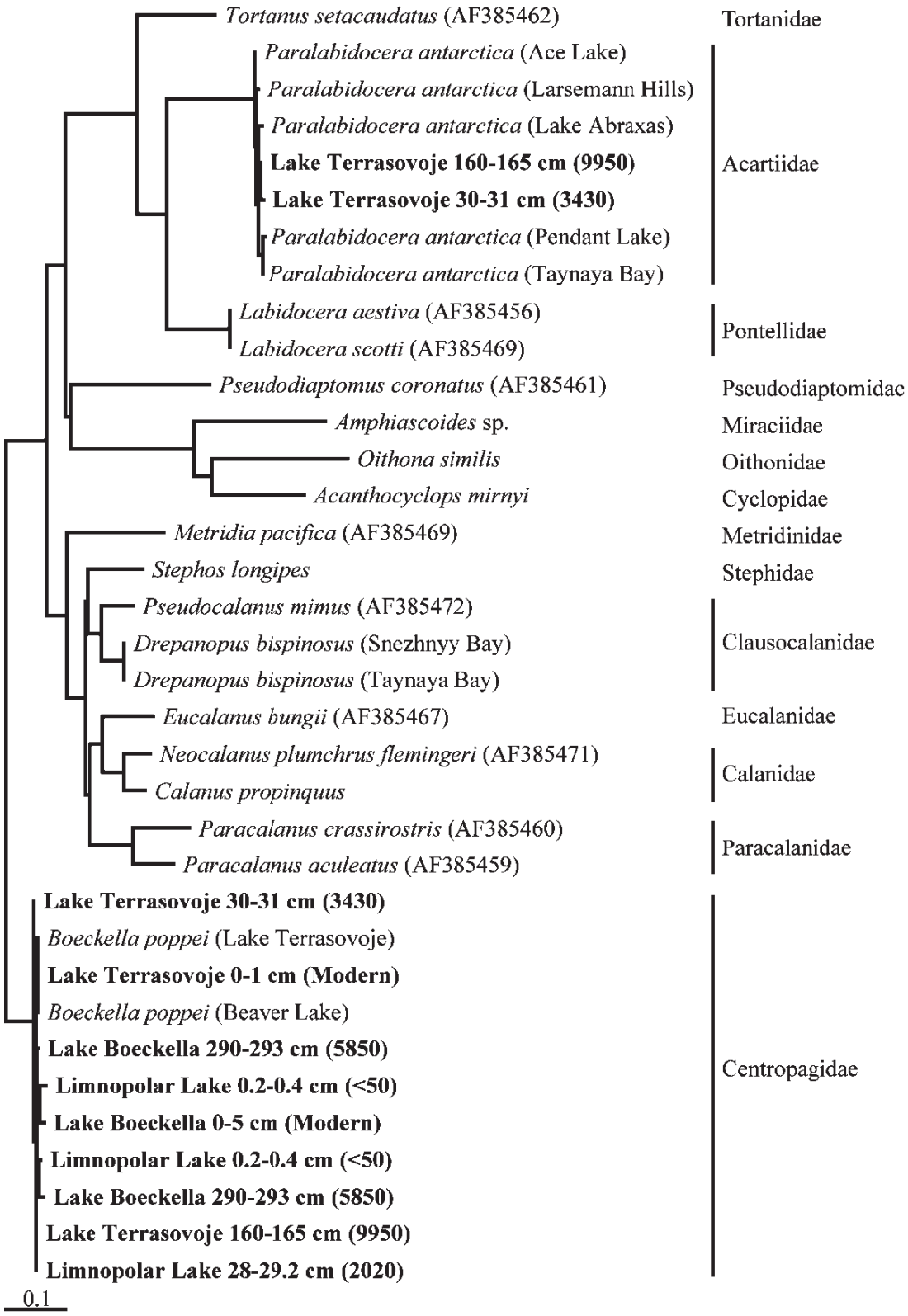

Fig. 2. A neighbor joining tree for partial large subunit rDNA sequences for 10 copepod species taken from GenBank, 14 sequences produced from copepod specimens identified in this study, and 10 sequences amplified from lake sediments (shown in bold, with estimated age [Table 1] in brackets). The families to which the copepod species belong are also shown. The tree shows the degree of sequence similarity between sequences derived from sediment samples and sequences derived directly from copepods.

Copepod sequences were also obtained from the sediments of Lake Boeckella and Limnopolar Lake. In each case the sequences grouped closely with those of modern *Boeckella poppei* from Lake Terrasovoje and Beaver Lake. This species is also present in Lake Boeckella and Limnopolar Lake today. No difficulties were encountered during processing of the Lake Boeckella samples that had been preserved with PEG.

The presence of copepods was confirmed by microscopy studies. Copepod eggs (generally hatched) and spermatophores identical to those of modern *Boeckella poppei* were found in all samples except those from Lake Reid. A second spermatophore type was observed in samples from the deepest sediment investigated from Lake Terrasovoje, which also contained abundant fecal pellets that were similar in shape to those recorded in sediment from Ace Lake in the Vestfold Hills, Antarctica (Cromer et al. 2005b). The pellets in the Ace Lake sediment were identical to those produced by modern *Paralabidocera antarctica* and were attributed to that species (Cromer et al. 2005b). Furthermore, fecal pellets were not recorded from the surface sediment of Lake Terrasovoje or the sediments of Lake Boeckella, in which *Boeckella poppei* has been abundant since the formation of the lake (J. Gibson, unpublished data). The presence of fecal pellets in the deeper Lake Terrasovoje sediments is therefore consistent with the occurrence of a second copepod species, possibly an Acartiid. The younger sediments from Lake Reid did not contain copepod remains, and no copepod species have been recorded from the water column or benthos of the lake (Dartnall 1995). Mandibles and spermatophores of an unknown copepod species, however, were observed in sediments from Lake Reid that were formed >50,000 y BP (Cromer et al. 2005a).

A major practical concern in studies of ancient DNA is the stability of the DNA itself, especially to hydrolysis and therefore irreversible degradation (Willerslev et al. 2004). It has been suggested that these problems are minimized for samples preserved in cold environments not exposed to UV radiation, where DNA might survive for up to 100,000 years (Wayne et al. 1999). Anoxic conditions, notably the presence of hydrogen sulfide, have been suggested as another factor enhancing preservation (Coolen and Overmann 1998), and adsorption of DNA to clay particles may also reduce DNase activity (Lorenz and Wackernagel 1987; Khanna and Stotzky 1992). Our results reinforce the conclusion (Coolen et al., 2004a, b) that the cold (typically <4° C) and anoxic conditions found in Antarctic lake sediments provide an excellent environment for the preservation of DNA.

A further, and perhaps more worrying, concern is the possibility of contamination, and the results of this and all studies claiming recovery of ancient DNA must be viewed critically with respect to this problem (Cooper and Poinar 2000). We are convinced that our successful isolation of DNA from lake sediments was not the result of contamination. Contamination could have occurred during sampling, during subsequent handling, or during the DNA extraction process. Modern sediment may have been smeared on the inside of the core tubes during collection, but careful isolation of sediment from the center of the core should have eliminated this mode of contamination. The identification of a sequence in the sediments of Lake Terrasovoje from a second species not currently found in the lake indicates that it is unlikely that contamination with recent sediment is the source of the DNA; if it were, the same sequence should have been recovered from the surface sediment. Contamination during subsequent handling cannot be entirely ruled out, but in this study, a sample that had undergone minimal postcollection handling (the frozen core from Lake Terrasovoje) returned the same sequences as that treated less rigorously. Finally, contamination could occur in the laboratory during extraction, PCR, and clone library development. Although not as stringent as those suggested by Cooper and Poinar (2000), the steps we employed to avoid contamination were quite rigorous and, from the absence of amplifiable DNA from all blanks, appeared successful. The precautions described by Cooper and Poinar (2000) should be taken if possible, but are probably not entirely necessary when dealing with nonhuman or nonbacterial targets where contamination is less likely and when amplification results can be confirmed by other methods, as we were able to do here with microscopy. No previous studies involving *Boeckella poppei* had been undertaken in our laboratory, so if the source of the sequence in the sediments was the initial extraction of modern example of this species, it should also have appeared in blanks as well as the samples from Lake Reid from which no copepod DNA was isolated. Similarly, if the source of the second sequence in Lake Terrasovoje was *Paralabidocera antarctica* extracted previously, this sequence should also have appeared in the surface sediments from Lake Terrasovoje and perhaps that from the other lakes. An important aspect is that the results were repeatable on fresh sediment sampled, extracted, amplified, and sequenced at later dates; if the sequences were the result of the laboratory contamination, the selective nature of the contamination was most unusual.

We are therefore confident that we have achieved the first successful isolation, amplification, and identification of metazoan DNA preserved in early-mid Holocene lake sediments. Our failure to extract and amplify DNA from the sediment core from Lake Reid provides further support for the method we have developed. The deeper sediments analyzed from this core contained copepod remains, but are over 50,000 years old (Hodgson et al. 2005). Therefore the DNA originally contained in them is likely to be far more degraded than that in the Holocene sediments. If we had been able to recover DNA from these Pleistocene sediments, either our methodology would be called into question, or a reassessment of the rate of DNA hydrolysis would have to be made. Our failure to amplify copepod DNA from the younger sediment sample was consistent with the absence of copepods from the modern lake (Dartnall 1995) and copepod remains from Holocene sediments (Cromer et al. 2005a).

We believe that this is the first record of successful extraction of DNA from PEG-preserved sediments. PEG is widely used in the preservation of modern samples to reduce DNA hydrolysis (e.g., Armstrong et al. 2002), and the isolation of DNA in this study is therefore not surprising. The successful amplification of DNA from each of the storage methods used for the samples in this study—freezing, storing at 4° C, and preservation with PEG—increases both the flexibility of sampling and storage protocols and the range of archived sediment samples that can be studied by this method. However, care should be taken with material stored at 4° C, as postcollection bacterial or fungal growth could occur that may degrade the metazoan DNA.

The Cop primers provided a 306-bp DNA fragment that was able to distinguish between the copepod species sequenced in this study and other species for which sequences were available through GenBank (Fig. 2). Although the relatively short amplification product may limit the elucidation of evolutionary events within the Copepoda, the aim of this study was to develop a screening method for identifying the presence of copepod species in ancient sediments, a task for which a small fragment appears ideal. Furthermore, choosing to amplify a relatively short DNA fragment increases the likelihood of success given the age of the sediments (Coolen and Overmann 1998). That the Cop primers were able to provide amplification products from sediments up to 9950 calibrated ^{14}C y BP (Table 1) indicates their suitability to this type of study. The use of short DNA sequences as species identification tools has gained considerable recent attention (Hebert et al. 2003; Tautz et al. 2003; Jarman 2004), and this study provides another example of the usefulness of this tool for both modern and ancient environments.

Our successful amplification of DNA from the lake sediments indicates that DNA is present in some form. There is no direct evidence, however, of what the form is; possibilities include dead animals, sperm cells in spermatophores, and unhatched eggs. A factor that may have contributed to the success of this work was that we targeted a group of organisms that produce diapause eggs that can survive long periods of dormancy (Hairston 1996). It is highly likely that the diapause eggs have mechanisms for preserving DNA, and that these mechanisms contribute to the longevity of copepod DNA in lake sediments. However, production of diapausing eggs does not occur in all families of the copepoda (Hairston and Cáceres 1996; Hairston and Bohonak 1998), and, if such eggs were the source of the DNA in this study, there may be a bias in this method against those species and families in which diapause egg production has not evolved.

Discussion

In this study we developed a method to extract, amplify, and sequence metazoan DNA from bulk lake sediments. Careful application of this and similar molecular genetic methods will provide a new suite of insights into the development of the biota of lakes and the response of the biota to changes in the habitat that result from local, regional, and global factors. We chose to target copepods in this study in part because these animals, which are important components of most lake ecosystems, leave few records in sediments and are rarely used in paleoecological studies. Copepod exoskeletons are only occasionally observed in sediments (Warner 1989; Rautio et al. 2000), and only difficult-to-identify eggs and spermatophores are generally found (Warner 1989). Our results indicate that use of DNA-based methods will be able to provide significant information about the development of copepod communities in lakes, especially in polar and subpolar zones.

The results obtained in this study highlight the potential of the method. While the recovery of DNA attributable to *Boeckella poppei* from Lake Terrasovoje sediments was not unexpected, the occurrence of an Acartiid in this lake was a considerable surprise. This species did not leave identifiable remains, and therefore its presence during the lake's history could have been overlooked if the molecular genetic method were not applied to the sediments.

Paleolimnology is a vibrant scientific field (Cohen 2003), and the results of our study indicate that more widespread use of molecular genetic techniques would be valuable. Even though classic microscopic analysis is still important for determination of abundance of zooplankton and other metazoan remains, molecular genetic screening of sediments could provide rapid identification of the species present that could both confirm the taxonomy indicated by microscopy and indicate the presence of otherwise cryptic species.

The DNA method could theoretically be made more quantitative by calibrating the PCR product concentration for each species of interest on the basis of per-unit mass of copepods. For targeted questions, such as the relative biomass of two or three copepod species, it would be relatively easy to get rough quantification. The precision would not be great, as signal strength would vary between individuals due to differing life stages, lipid content, and other factors, but this variation might not be worse than quantification by more traditional methods. This is a rapidly advancing field and we expect that DNA-based analysis of sediment samples will eventually provide accurate identification and quantification for a wide range of taxa, including groups like copepods that are not commonly used in traditional paleolimnological studies.

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