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The Holocene 2008; 18; 1003
DOI: 10.1177/0959683608093540

The Holocene 18,6 (2008) pp. 1003–1006

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Received 18 June 2007; revised manuscript accepted 21 February 2008

A HOLOCENE RESEARCH REPORT

Abstract: This paper describes our recent attempt to isolate and analyse DNA from old plant remains of the common peatmoss *Sphagnum fuscum* retrieved from a peat core collected in the mire Fuglmyra, in central Norway. DNA was recoverable and usable from subfossilized (10–450 years old) plant remains of the peatmoss. A chloroplast (*trn*L) and two nuclear (ITS2 and RAPDf) regions were co-amplified from 80 samples of different ages. The RAPDf region was the only variable one with three different haplotypes found among five samples. Comparison of the ancient sequences with modern sequences found in the extant population occurring at the same site ascertained a genetic link between modern and fossil samples of this species. This retrieval of ancient DNA from subfossilized moss remains isolated from peat cores has important implications for the palaeoecology of peatmosses by allowing direct estimates of plant population dynamics in space and time.

Key words: Ancient DNA, *trn*L, ITS2, RAPDf, *Sphagnum*, peatlands, population dynamics, palaeoecology, Norway, Holocene.

Introduction

Studies on plant ancient DNA (aDNA) are difficult to carry out, mainly because of the poor DNA preservation conditions, since biological material is quickly decomposed and degradation of DNA in aerated conditions is rapid (Pääbo *et al*., 2004). This is particularly true in plants, where well-preserved hard tissues are more difficult to find than in animals. Moreover, because most wood cells lack viable nuclei and plastids, their genetic analysis is often hampered by technical difficulties. Studies of plant aDNA have therefore lagged well behind those of animals, despite the wealth of subfossil material available for molecular studies, from wood to leaves, needles and pollen. Nevertheless, over the last two decades, aDNA has been successfully extracted from different plant material (for a review see Gugerli *et al*., 2005), including pollen (Suyama *et al*., 1996; Parducci *et al*., 2005; Bennett and Parducci, 2006).

Northern peatlands are wetlands with a biomass-accumulating peatmoss surface layer. Peat is a type of soil that consists of dead organic matter, mainly from plants, that has accumulated over thousands of years because of the low decomposition rate and the anoxic environment at shallow depth below the moss surface. The peatmosses (*Sphagnum* spp.) of the Northern Hemisphere contribute to the accumulation of peat by keeping a high water-table and by having a slow decomposition rate. This is why the genus *Sphagnum* is responsible for more fixed carbon on the surface of Earth than perhaps any other single plant genus (Clymo, 1998). In addition, the accumulated peat is an archive for biological material and has often been used in peat stratigraphy (palynology), where the identification of subfossil plant material has helped in the understanding of the developmental history of mires (eg, Barber, 1981).

The peatmoss species *Sphagnum fuscum* (Schimp.) Klinggr. dominates large areas of northern peatlands. Most of the peatlands in Scandinavia originated after the last glaciation during the Boreal and Atlantic periods (9000–5000 yr BP; Solelm, 1986) and it is believed that colonization events at these early stages had profound effects on the spatial genetic structure found today in modern populations, even at a fine local scale. Because of extensive clonal growth, moss populations may be founded by one or a few genets, which subsequently dominate in the area (eg, Cronberg *et al*., 2006). Some authors, however, have suggested that new establishment occurs continuously, giving rise to high haplotype diversity within sites (eg, Cronberg, 1996, 2002; Stenøien and Såstad, 1999; van der Velde *et al*., 2001). Gunnarsson *et al*. (2007) recently investigated

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10.1177/0959683608093540

the genetic structure of a modern population of *S. fuscum* in central Norway and analysed the sequences of three anonymous, likely nuclear, DNA regions. They found that haplotypes were grouped in two major types, and that revealed microhabitat differentiation along the groundwater-table and pH gradients. They also suggested that vegetative growth and asexual reproduction from gametophyte fragments were the two dominant reproductive modes in the population.

This paper describes our recent attempt to isolate and analyse DNA from old plant remains of *S. fuscum* from a peat core sampled in Norway at the same site described in Gunnarsson *et al*. (2007). The aim was to recover DNA from subfossilized plant remains of this species and to analyse spatial and temporal genetic variation at the same site.

Materials and methods

A 1.3 m long peat core (11 cm in diameter) was retrieved in September 2004 from a large area dominated by *S. fuscum* in an ombrotrophic part of the Fuglmyra mire, Sør-Trøndelag, central Norway (63°16′N, 10°25′E; 180 m a.s.l.). After collection the bottom of the core was locked and the core was brought to the laboratory and frozen at –20°C. Sample preparation and DNA isolation were carried out in a clean laboratory in Trondheim, Norway, while PCR amplification and sequencing were conducted in a second laboratory in Uppsala, Sweden. To avoid contamination during sampling we followed rigorous contamination control measures to reduce the risk. We took shoots only from the internal part of 5 cm deep discs previously sawn from the frozen core. Sixteen discs and five shoots per disc (80 samples in total) were sampled and carefully washed in sterile water under a dissecting microscope. Total DNA was isolated from single shoots with the DNeasy Plant Mini Kit (QIAGEN). From these 80 samples we amplified short internal fragments of the chloroplast *trn*L gene, the nuclear ITS2 region, and the internal variable fragment of one anonymous, presumably nuclear, region designated RAPDf (Shaw *et al*., 2003). Designing the primers for amplification and sequencing of the three fragments was a critical step for targeting informative fragments shorter than 150 base pairs (bp). Primer design was conducted using the computer program OLIGO (National BioScience) and was based on a multiple alignment of the *S. fuscum* DNA sequences retrieved from GenBank. Sequences of the primer pairs used are presented in Table 1.

We used nested PCR to reduce the contamination in products due to the amplification of unexpected primer binding sites and to increase the amount of the target DNA to be sequenced. Two sets of primers were used in two successive runs of polymerase chain reaction, with the second set intended to amplify the internal shorter fragment within the first run product. We ran multiple PCR amplifications of the three fragments together (*trn*L, ITS2 and RAPDf) using the first set of primers (Table 1) in a thermal cycler (GeneAmp PCR System 2400, Applied Biosystems) in a 10 µl reaction volume containing 0.5 µl of extracted DNA, 0.2 µM each primer and 5 µl of QIAGEN Multiplex PCR Master Mix (QIAGEN). Forty PCR cycles were run with cycle conditions of 94°C for 30 s, 58°C for 90 s and 72°C for 90 s, an initial denaturating step at 95°C for 15 min, and a final incubation step at 72°C for 10 min. After PCR, 5 µl of the product reaction was electrophoresed in 2% agarose gel, stained with ethidium bromide and visualized by UV transillumination. The PCR products that visualized the band of correct size on agarose gel were used as a template in the second stage of the nested amplification using internal primers. Each second PCR amplification for the three fragments was performed separately using a thermal cycler (iCycler, Bio-Rad Laboratories) under the following conditions: initial activation at 98°C for 30 s, then 35 cycles of denaturation at 98°C for 10 s, annealing at 50°C (*trn*L) or 65°C (ITS2 and RAPDf) for 10 s, followed by a final incubation at 72°C for 5 min. The volume of the reaction mixture was 15 µl containing 0.1 µl of the first PCR products, 3 µl of 5×Phusion HF Buffer (Finnzymes Oy), 0.2 µM each dATP, dCTP, dGTP and dTTP, 0.5 µM each internal primer and 0.15 µl of Phusion DNA Polymerase (Finnzymes Oy). Five µl of PCR product was separated by electrophoresis on 2% agarose gel to evaluate the results of the second PCR amplifications. After the second amplification all PCR products were purified using ExoSAP-IT (GE Healthcare) and DNA fragments were sequenced. To test the repeatability of the amplifications we repeated the analyses at least twice from the first-PCR step on all the DNA isolates that produced expected fragments after the second PCR. Sequencing was conducted using upper primers (Table 1) and sequences were resolved on a MegaBASE 1000 DNA Sequencing System (GE Healthcare).

Results and discussion

On the whole, successful amplifications and sequencing analysis in the *S. fuscum* samples were achieved in 5/80, 1/80 and 5/80 of the samples analysed for the *trn*L, the ITS2 and the RAPDf regions, respectively (Table 2). Repeated amplifications from the same isolate gave consistently identical results (sequences). The RAPDf

Table 1 Primer sequences and their use in the different polymerase chain reactions

Region	Primer	Use	Sequence (5'-3')	PCR product length (bp)
*trn*L	90U[a]	First PCR	CCCAACGAGAAATATTACTTTGA	170
	239L[a]	First and nested PCR	CTCCGAGATTTCTATCTGGTG	150
	110U[a]	Nested PCR and sequencing	TGACTTTAGATCTTATACG	
ITS2	410U[b]	First PCR	TTGCAGAATTCCGCGAATCATC	227
	617L[b]	First PCR	GATCACCTGGTCACGGTCCC	
	461U[b]	Nested PCR and sequencing	CTTGTCCCGAGGGCATGTC	136
	575L[b]	Nested PCR	CATACATTTCAGCCGACCGATG	
RAPDf	204U[c]	First PCR	TGAAGTGATCAAATCAAGAGCA	181–206
	393L[c]	First PCR	AAGCGCTCAGCAGCAAT	
	223U[c]	Nested PCR and sequencing	GCAATGCTCAAGAGGTATGTAT	155–180
	382L[c]	Nested PCR	CAGCAGCAATGTTACCAAGTA	

[a]Positions of the 5' end base are numbered according to the *S. fuscum* chloroplast *trn*L gene sequence (Shaw *et al*., 2003; Accession No. AY297999), [b]ITS1, 5.8S, ITS2 and 26S sequences (Shaw *et al*., 2003; Accession No. AY298465) and [c]RAPD-F sequence (Shaw *et al*., 2003; Accession No. AY346843).
U, upper primer; L, lower primer

Table 2 Haplotypes found at different depths for the three investigated DNA regions and number of successful amplifications

Sample	Depth in the core (cm)	Approx. age*	*trn* L		ITS2		RAPDf	
			Haplotype	Replicates	Haplotype	Replicates	Haplotype[c]	Replicates
#278	10	10	*S. fuscum*[a]	1	*S. fuscum*[b]	1	1–3	1
#210	25	50	–	–	–	–	14–16	1
#208	25	50	*S. fuscum*[a]	1	–	–	–	–
#207	25	50	*S. fuscum*[a]	2	–	–	14–16	2
#205	25	50	*S. fuscum*[a]	1	–	–	1–3	3
#201	65	450	*S. fuscum*[a]	2	–	–	13	3

*Ages of top samples approximated from a normal peat accumulation curve (Ohlson and Dahlberg, 1991). For samples deeper than 25 cm the age was calculated assuming a peat deposition rate of 1 mm ×year^{-1}.
[a]Identical to the extant *S. fuscum* chloroplast *trn*L sequence found in Shaw et al. (2003); Accession No. AY297999.
[b]Identical to the extant *S. fuscum* ITS2 sequence found in Shaw *et al*. (2003); Accession No. AY298465.
[c]Haplotypes numbered as in Gunnarsson *et al*. (2007)

region was the only variable of the three loci analysed (one deletion and one polymorphic nucleotide sites) and revealed three different haplotypes while the *trn*L and ITS2 regions showed no variation. The sequences obtained were identical to the published sequences of *S. fuscum* (Table 2). Only one out of the three RAPDf haplotypes found in the peat core (haplotype 13) was found among modern samples at exactly the same site (Gunnarsson *et al*., 2007). On the other hand, the most common haplotype found among modern samples (haplotype 6) was not detected in the peat core, suggesting that the haplotype structure has changed at this site through time.

The technique of multiple amplifications gave valuable information about DNA preservation in the samples. According to Dongya and Speller (2006), there is an inverse relationship between the length and the amount of DNA templates present in a sample: the longer the fragment the fewer molecules are preserved intact. In this study, we succeeded in amplifying from six out of 80 samples tested in at least one of the three regions analysed. Our results suggested that there was an uneven amplification of the three DNA fragments during the first multiple PCR amplification. This was likely due to the combined effect of the age of the DNA, the efficiency of the primer pair and the length of the fragment amplified. As expected, the longest fragment (ITS2, 227 bp) was more difficult to amplify compared with the other two shorter fragments (*trn*L, 170 bp; RAPDf, 181–206 bp) and the youngest sample (#278) was the only that gave positive amplifications for all three fragments (including the ITS2 nuclear region) (Table 2).

In this study we followed several criteria in order to control DNA contamination from both extant and ancient DNA. The primers used worked specifically on the DNA of *Sphagnum* species and the sequences of the RAPDf region could differentiate different species within the genus. The multiple amplification technique makes the detection of contamination among samples easier when multiple DNA fragments indicate two different species or subspecies. In our case, no peatmoss species other than *S. fuscum* were present in the peat core, however if erroneous amplifications started from DNA present in the peat from other taxa they would yield distinct sequences or no sequences at all. To further exclude the possibility that the results were due to amplification starting from PCR amplicons, we worked in physically isolated areas. Manipulations of peat samples and DNA isolation were performed in Norway and all manipulation of the DNA extracts prior to PCR were performed in Sweden in a room isolated from the one with work involving PCR and DNA sequencing, where extant DNA and/or PCR products were never introduced. Here, we regularly sterilized laboratory equipment and reagents, and during analyses we wore dedicated protective clothing. We also used negative controls throughout our experimentation (during first and second rounds of PCR) and no PCR products were obtained from them. Finally, to reliably investigate nucleotide variation and to exclude variation due to PCR misincorporations, we repeated all PCR amplifications from the same isolate more than two times and consistently obtained the same results (DNA sequences).

The results presented here are not in themselves adequate to demonstrate how the *S. fuscum* population has changed through time. Extension of this work to additional sites in central Norway as well as analyses of older material should enable a more detailed picture of population-level spatial dynamics over time, which should make it possible to understand more precisely what happens genetically when this species spread over this area within relatively short periods of time.

Acknowledgements

This study was financed by grants from the Japan Society of the Promotion of Science (No. 17200050) to YS, the Research Council of Norway (project no. 159589/V40), the Royal Swedish Academy of Science and the Extensus Foundation to UG, and the Carl Trygger's Foundation to LP.

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