The oldest sequenced fungal herbarium sample

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Short Communications

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Biological studies dealing with molecular data use fresh specimens by preference, but in taxonomy molecular data must sometimes be obtained from herbarium material, particularly for type material or for rare taxa not collected recently. Whereas modern taxonomic studies use genotype data from rather recent samples for confirmation of phenotype-based taxa, the names of such taxa may be based on old type specimens whose genotype has not been investigated. We decided to attempt to sequence old herbarium samples in order to solve some nomenclatural problems in modern taxonomy.

DNA degrades gradually over time, so amplifying DNA from old specimens is difficult. Fragmentation is one of the main degradation processes but rather short DNA regions may be successfully PCR-amplified and sequenced even from old material (e.g., Ubaldi *et al.* 1998). Old and fossil DNA may be well-preserved in materials such as amber (DeSalle *et al.* 1992; Cano & Borucki 1995), and also sometimes in herbarium specimens (Rogers & Bendich 1985). The oldest successfully sequenced herbarium specimen dates from more than 200 years ago and is from a collection of the vascular plant *Phaulopsis talbotii* S. Moore (Andreasen *et al.* 2009). Successful PCR amplification of a 100 year-old specimen of the liverwort *Bazzania trilobata* (L.) Gray is a record for bryophytes (Jankowiak *et al.* 2005). Sequences have also been obtained from very old herbarium samples of some plant pathogens from Stramenopila: May & Ristaino (2004) succeeded with 159 year-old *Phytophthora infestans* (Mont.) de Bary, and Telle & Thines (2008) with a 129 year-old oomycete. In lichen-forming fungi herbarium specimens up to 35 years old have been used routinely for successful DNA extraction (Grube *et al.* 1995). Recently, Sohrabi *et al.* (2010) successfully sequenced ITS regions from a 75 year-old herbarium specimen of *Aspicilia aschabadensis* (J. Steiner) Mereschk., previously the record for old lichen specimens. In our study, we assess PCR amplification and sequencing of ITS regions (rDNA) from herbarium samples of lichens up to 151 years old; a collection from 1859 sequenced in 2010. The 151 year-old, successfully sequenced lichen mycobiont is a record not only for lichen-forming but for all fungi.

We isolated DNA using the CTAB-based protocol from Aras & Cansaran (2006) with the following minor modifications. Five to ten fruit-bodies of each lichen were put in 1·5-ml tubes, frozen in liquid nitrogen and ground to a fine powder using a pestle. All centrifugation steps were carried out at 14 600 × ***g***. Isopropanol and ethanol solutions were kept at −20°C until used. The DNA pellet was air-dried in a dry bath to remove residual ethanol, resuspended in 25–30 µl of TE buffer, treated with 15 µg of RNase A (Fermentas) and stored at −20°C until used. The ITS region was amplified using primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990). PCRs were performed in a reaction mixture containing 1µl of genomic DNA, 2·5 mM $MgCl_2$, 0·2 mM of each dNTP, 0·2 µM of each primer (Invitrogen), 1·25 U *Taq* polymerase (Top-Bio, Praha, Czech Republic) in the manufacturer's buffer, and sterile water to make up to a final volume of 25 µl. A negative control was included in PCR assay, and no recent lichen DNA samples were utilized for PCRs together with old samples at the same time. Cycling parameters followed Ekman (2001).

ITS nrDNA sequences of full lengths (560–850 bp; the difference in lengths is mainly due to the *c.* 200 bp indel at the beginning of the region sequenced) were obtained from 12 of 20 mycobionts of *Caloplaca* (lichenized fungi of *Teloschistaceae*),

collected between 1859 and 1988 (Table 1). We succeeded with three of four samples collected in the 19th century, with two of six samples from 1900–1950, and with seven of ten samples collected after 1950.

The most similar BLAST hits to our sequences (Table 1) were in accordance with our knowledge on phylogeny and morphology of the taxa analyzed. Among the four oldest herbarium specimens successfully sequenced, the 1859 specimen is the holotype of *Caloplaca conversa* (Kremp.) Jatta. Its sequence is most similar to our *C. conversa* sample from Iran, HQ611273 (94·4% nucleotide identity). The 1875 specimen of *C. duplicata* (Vain.) H. Olivier is 99·6% identical to our sequence of a morphologically similar specimen of *C.* cf. *xerica* Poelt & Vězda, HQ611274, from Tatarstan. The 1882 specimen is the holotype of *C. percrocata* (Arnold) J. Steiner and shows 96·4% similarity with *C. albopustulata* Khodos. & S. Y. Kondr., EU192150. The 1911 specimen was identified by us as *C. fuscoatroides* J. Steiner and shows 97·5% similarity with the haplotypes of *C. ceracea* J. R. Laundon from 1964 and 1970, a species which is probably conspecific with *C. fuscoatroides*. The sequences obtained were included in Bayesian molecular analyses, which support identifications of sequences using the BLAST search.

It is probable that the degradation of DNA sometimes slows enough to allow sequencing of old herbarium material of lichens. Nevertheless, DNA extraction is probably a crucial step for successful PCR amplification (Telle & Thines 2008). Based on our results, the CTAB method seems to be suitable for obtaining DNA from herbarium specimens. It has a clear advantage of providing a relatively high yield of isolated DNA which is likely to be critical when processing old samples with partially degraded DNA. Besides methods using reagents such as CTAB or SDS for lysis of cell membranes and selective DNA precipitation, various commercially available DNA kits involving DNA binding on silica spin-columns are widely used in processing lichen samples; they are also promising for old herbarium specimens (e.g. Sohrabi *et al.* 2010). Telle & Thines (2008) analyzed oomycetes from old herbarium specimens using several extraction methods and concluded that most CTAB-based protocols and commercial DNA kits gave sufficient results, although DNA yield varied to some extent. An advantage of commercial kits over other extraction methods is the higher DNA purity. On the other hand, DNA yields of commercial kits are lower than those obtained by CTAB protocol and its modifications (Niu *et al.* 2008; Telle & Thines 2008).

According to the results of Soltis & Soltis (1993) and Jankowiak (2005), it seems more likely to obtain successful PCR amplification of ancient DNA in regions up to 500 bp. Nevertheless, we successfully amplified ITS regions which were considerably longer, between 560–850 bp. This could be due to the multi-copy nature of the rDNA, which facilitates PCR amplification, and makes it feasible even from ancient samples (Álvarez & Wendel 2003).

When working with old material, much care must be taken in all laboratory procedures especially during DNA extraction and PCR setup. It is also essential to check the sequences obtained for possible contamination. Gutiérrez & Marín (1998) warn against the risk of contamination of samples causing false positive results, for example in the case of sequences from amber samples (Cano *et al.* 1993) or from glacier-covered subfossil *Umbilicaria* samples (DePriest *et al.* 2000). In order to avoid uncertainties, taxonomic identities of sequences obtained by ourselves were further analyzed by BLAST similarity search, which confirmed that the DNA of the target organism was correctly amplified (Table 1).

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TABLE 1. *List of herbarium samples used for the sequencing experiment. Samples successfully ITS sequenced are highlighted in bold*

Herbarium name	Current name	Specimen data	Year of collection	GenBank accession	Closest Blast (% pairwise nucleotide identity)
Callopisma conversum	*Caloplaca conversa* (Kremp.) Jatta	Germany, Algau in Alps, *Rehm* (M-0012425, holotype)	1859	HQ234597	*C. conversa*, HQ611273 (94·4%), Iran, CBFS JV5538
Lecanora duplicata	*Caloplaca duplicata* (Vain.) H. Olivier	Finland, Karelia australis, *E. Lang* (TUR-V-7513)	1875	HQ611272	*C.* aff. *xerica* Poelt & Vězda, HQ611274 (99·6%), Tatarstan, LE
Lecanora helsinkiensis nom. ined.	*Caloplaca soralifera* Vondrák & Hrouzek	Finland, Helsinki, *Vainio* (TUR-V)	1876	–	
Blastenia arenaria* var. *percrocata	*Caloplaca percrocata* (Arnold) J. Steiner	Italy, Südtirol, *Arnold* (M- 0102293, lectotype)	1882	HQ234598	*C. albopustulata* Khodos. & S.Y. Kondr., EU192150 (96·4%)
Caloplaca spalatensis	*Caloplaca spalatensis* Zahlbr.	Croatia, Split, *J. Baumgartner* (W, holotype)	1900	–	
Caloplaca cerina var. *areolata*	*Caloplaca areolata* (Zahlbr.) Clauzade	Montenegro, Herceg Novi, *J. Baumgartner* (W, holotype)	1903	–	
Blastenia viperae	*Caloplaca herbidella* (Hue) H. Magn.	Croatia, Pelješač Peninsula, *J. Baumgartner* (W, holotype)	1906	–	
Caloplaca fuscoatroides	*Caloplaca fuscoatroides* J. Steiner	Greece, Delos, *J. Steiner* (W, topotype)	1911	HQ234599	*C. ceracea* J.R. Laundon, HQ234600, HQ234603 (97·5%)
Caloplaca diphiodes var. *helygeoides*	*Caloplaca diphyodes* (Nyl.) Jatta	Sweden, Torne Lappmark, *A. H. Magnusson* (GZU)	1919	–	
Caloplaca lactea var. *tunispora*	*Caloplaca ferrarii* s. lat. clade 1, *sensu* Vondrák *et al.* (2011)	Russia, Astrakhan region, *Tomin* (LE)	1926	HQ234605	*C. ferrarii* s. lat. HQ234604 (97·9%)
Caloplaca caesiorufa	*Caloplaca ceracea* J.R. Laundon	Great Britain, Forfar, *P.W. James* (BM-22085)	1964	HQ234600	*Caloplaca ceracea* J. R. Laundon, HQ234603 (100%)
Caloplaca atroflava var. *submersa*	*Caloplaca* cf. *percrocata* (Arnold) J. Steiner	Switzerland, Graubünden, *J. Poelt* (GZU)	1967	HQ234601	*C.* aff. *xerica*, HQ611275 (91·8%), Czech Republic, CBFS JV7618
Caloplaca marina	*Caloplaca ora* Poelt & Nimis	France, Corse, *J. Lambinon, Y. Rondon & A. Vězda* (W-15494, isotype of *C. ora*)	1969	HQ234602	*C. confusa* Vondrák, Říha, Arup & Søchting, EU563457 (99·3%)
Caloplaca albolutescens	*Caloplaca ceracea* J.R. Laundon	Great Britain, Anglesey, *P. W. James* (BM-6656)	1970	HQ234603	*Caloplaca ceracea*, HQ234600 (100%)
Caloplaca lactea	*Caloplaca ferrarii* s. lat. clade 1, sensu Vondrák *et al.* (2011)	Kirgizstan, Tian Shan Mts, *L. Bredkina* (LE)	1970	HQ234604	*C. ferrarii* s. lat. HQ234604 (97·9%)
Caloplaca furax	*Caloplaca furax* Egea & Llimona	Spain, Cañada del Conejo, *J. M. Egea* (GZU, isotype)	1978	–	
Caloplaca aetnensis	*Caloplaca aetnensis* B. de Lesd.	Spain, Catalania, *Hladun & Gómez-Bolea* (BCN)	1980	–	
Caloplaca atroflava* var. *submersa	*Caloplaca atroflava* (Turner) Mong.	Italy, Sardinia, *P.L. Nimis & J. Poelt* (GZU)	1985	HQ234606	*C. atroflava*, HQ611276 (96·4%), USA, CBFS 7494
Caloplaca ceracea	*Caloplaca ceracea* J.R. Laundon	Great Britain, Wales, *A. Orange* (BM-22083)	1988	–	
Caloplaca elegantissima	*Caloplaca elegantissima* (Nyl.) Zahlbr.	Namibia, Swakopmund, *O. L. Lange* (GZU)	1988	HQ234607	*Caloplaca syvashica*, HM582201 (82·4%)

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