A peer-reviewed version of this preprint was published in PeerJ on 26 May 2020.

View the peer-reviewed version (peerj.com/articles/9109), which is the preferred citable publication unless you specifically need to cite this preprint.

Saługa M. 2020. At the crossroads of botanical collections and molecular genetics laboratory: a preliminary study of obtaining amplifiable DNA from moss herbarium material. PeerJ 8:e9109
https://doi.org/10.7717/peerj.9109

At the crossroads of botanical collections and molecular genetics laboratory: testing methods to obtain amplifiable DNA from moss herbarium material

Marta Saługa Corresp. 1

1 W. Szafer Institute of Botany, Polish Academy of Sciences, Lubicz 46, 31-512 Kraków, Poland

Corresponding Author: Marta Saługa
Email address: m.saluga@botany.pl

Background. Museum collections, including herbarium specimens, are considered an invaluable source of DNA. They constitute a source of a precious commodity, particularly when it is difficult to obtain living material of rare species, or extant populations occurred only in hard to access geographical territories. It is apparent that herbaria should be directly linked with molecular genetics laboratories making them a quick, open-source for molecular projects. However, herbarium DNA is inherently characterised by high degradation and chemical modifications such as the presence of various secondary compounds. A wide range of DNA molecular techniques dedicated to the preserved plant material has been published so far. However, contrasting with a general interest in the application of molecular analyses in moss biology, no comprehensive assessment of DNA isolation and amplification methods from moss herbarium material, is available. **Methods.** To assess the feasibility of using DNA from moss herbarium specimens, we have tested and compared the silica column-based method and three variants of CTAB-based DNA extraction protocol. We used herbarium collections of twenty-five moss species collected between 1979 and 2013 and specifically focused on austral polar regions to assess the potential of herbarium as a source of biological material from geographical regions of difficult and restricted access. **Results.** Here, we present an optimized CTAB-based approach which effectively suppresses inhibitors in the herbarium DNA as was measured by amplification success. In this report, DNA purity and the length of the target genetic region are the fundamental agents which drive the successful PCR reaction. Conversely, the specimen age seems to be less relevant. Moreover, the size distribution of the DNA fragments extracted using Qiagen protocol is shown to be comparable to our original CTAB-based approach. Our modified CTAB-based method provides a high-purity genomic DNA allowing efficient downstream amplification. It is not outcompeted by the column-based method and appears as a method of choice in molecular studies of moss herbarium material.

At the crossroads of botanical collections and molecular genetics laboratory: testing methods to obtain amplifiable DNA from moss herbarium material

Marta Saługa

W. Szafer Institute of Botany Polish Academy of Sciences, 46 Lubicz Str., 31-512 Kraków, Poland

Corresponding Author:
Marta Saługa
46 Lubicz Str., 31-512 Kraków, Poland
Email address: m.saluga@botany.pl

Abstract

Background. Museum collections, including herbarium specimens, are considered an invaluable source of DNA. They constitute a source of a precious commodity, particularly when it is difficult to obtain living material of rare species, or extant populations occurred only in hard to access geographical territories. It is apparent that herbaria should be directly linked with molecular genetics laboratories making them a quick, open-source for molecular projects. However, herbarium DNA is inherently characterised by high degradation and chemical modifications such as the presence of various secondary compounds. A wide range of DNA molecular techniques dedicated to the preserved plant material has been published so far. However, contrasting with a general interest in the application of molecular analyses in moss biology, no comprehensive assessment of DNA isolation and amplification methods from moss herbarium material, is available.

Methods. To assess the feasibility of using DNA from moss herbarium specimens, we have tested and compared the silica column-based method and three variants of CTAB-based DNA extraction protocol. We used herbarium collections of twenty-five moss species collected between 1979 and 2013 and specifically focused on austral polar regions to assess the potential of herbarium as a source of biological material from geographical regions of difficult and restricted access.

Results. Here, we present an optimized CTAB-based approach which effectively suppresses inhibitors in the herbarium DNA as was measured by amplification success. In this report, DNA purity and the length of the target genetic region are the fundamental agents which drive the successful PCR reaction. Conversely, the specimen age seems to be less relevant. Moreover, the size distribution of the DNA fragments extracted using Qiagen protocol is shown to be comparable to our original CTAB-based approach. Our modified CTAB-based method provides

a high-purity genomic DNA allowing efficient downstream amplification. It is not outcompeted by the column-based method and appears as a method of choice in molecular studies of moss herbarium material.

Introduction

In recent years, assessing techniques of DNA isolation and sequencing from herbarium plant material has gained broad interest (e.g. Rogers & Bendich, 1985; Goff & Moon, 1993; Savolainen et al., 1995; Porebski, Bailey & Baum, 1997; Blattner, 1999; Drábková, Kirschner & Vlĉek, 2002; DeCastro & Menale, 2004; Jankowiak, Buczkowska & Szweykowska-Kulinska, 2005; Cota-Sánchez, Remarchuk & Ubayasena, 2006; Telle & Thines, 2008; Soni & Kumar, 2009; Lehtonen & Christenhusz, 2010; Staats et al., 2011; Särkinen et al., 2012; Staats et al., 2013; Shepherd & Perrie, 2014; Shepherd, 2017; Do & Drábková, 2018; Höpke et al., 2019). The primary aim of such technical investigations is to establish appropriate methods of efficient DNA analysis from preserved plant specimens, taking into account the specificity of such source material. This specificity includes especially DNA damage in historical material, notable degradation into short fragments as well as chemical modifications which may result in the poor success of genomic DNA isolation and subsequent PCR amplification of DNA fragments (Staats et al., 2011). Accordingly, the success rate of DNA amplification based on fresh samples is substantially higher than that based on herbarium specimens (Shepherd & Perrie, 2014).

While DNA analysis from herbarium material presents technical difficulties, sharing empirically tested innovative molecular protocols for biological collections is of major importance (e.g., Lavoie, 2013). Exploration of a wealth of materials deposited in biological museum collections has an invaluable potential for taxonomic and phylogenetic studies but also biogeographical and environmental research. Owing to herbaria sources, there is also an opportunity for collecting large data set including specimens of various taxonomic position, habitats, and geographical distribution patterns, without the need for undertaking field expeditions. Moreover, herbarium collections are of critical importance in cases where adequate taxonomical and/or geographical sampling of fresh material in the field is difficult or impossible. Here, biological collections from hardly accessible regions could especially play an indispensable role.

In spite of growing accumulation of published technical experience based on empirical tests, it is important to notice that success of extraction and PCR amplification of herbarium DNA may be strongly affected by factors specific for taxonomical groups, taxa and even specimens (Särkinen et al., 2012). For some taxonomic groups, focused reports of step-by-step improvements of herbarium DNA extraction and amplification methods are still scarce. This is the case of bryophytes, which contrasts with current interest and a central position of molecular studies in moss biology. Collections of mosses deposited in herbaria worldwide may provide an excellent source for DNA based bryological studies.

Mosses, being organisms well adapted to many environmental constraints, are often key components of flora in inaccessible and ecologically inhospitable environments. One extreme

example is Antarctic biome, locked-in ice continent with only 0.18% to 0.32% of the area being ice-free. Altogether, 115 species of mosses are known from Antarctica (Ochyra, Lewis Smith & Bednarek-Ochyra, 2008; Ochyra, Bednarek-Ochyra & Lewis Smith, 2008; Sollman, 2015; Jiménez & Ochyra, 2017; Ronikier et al., 2018). However, botanical exploration of the Antarctic continent and the maritime Antarctic islands is highly restricted due, among others, to substantial logistic limitations. While herbaria can be very important for studies focused on this area, the number of molecular studies using herbarium specimens from polar regions of the Southern Hemisphere is very low. Lavoie (2013) found only three articles that used herbarium specimens from Antarctica for documenting biogeographical patterns or environmental changes. Since then, we have found only a handful of further studies dealt with herbarium moss material from Antarctica including our recent contributions (Pisa et al., 2014; Biersma et al., 2018a; 2018b; Ronikier et al., 2018; Saługa et al., 2018). On the other hand, there is a clearly growing interest in research focused on mosses from the high-latitude ecosystems of the Southern Hemisphere and related to their biogeography, evolutionary history and ecology (e.g. Convey & Lewis Smith, 2006; Peat, Clarke & Convey, 2007; Cannone & Seppelt, 2008; Ochyra, Bednarek-Ochyra & Lewis Smith, 2008; Ochyra, Lewis Smith & Bednarek-Ochyra, 2008; Li et al., 2009; Van der Putten et al., 2009; Hills, Stevens & Gemmill, 2010; Cannone, Convey & Guglielmin, 2013; Kato et al., 2013; Fraser et al., 2014; Pisa et al., 2014; Biersma et al., 2017; Rankin et al., 2017; Waterman et al., 2017; Biersma et al., 2018a; 2018b; Fraser et al., 2018; de Freitas et al., 2018; Ronikier et al., 2018; Saługa et al., 2018).

The paper aims to comprehensively examine the methods of improving the use of moss herbarium material for Sanger DNA sequencing. We present a test of DNA isolation methods for herbarium moss specimens and assess their utility. Then, we test the obtained DNA isolates as sources for successful PCR amplification of selected target regions in the length range of no more than 100-150 bp to approximately 1500 bp from low concentrated samples (c. 2,0 ng/µL) of up to 39 years old herbarium specimens. In our report, we consider in particular the age of specimens used, DNA quality and quantity, target amplicon size and DNA severe fragmentation. To estimate a direct relevance for biological studies focused on difficult geographical territories, we deliberately based our analyses on moss materials from the hardly accessible austral polar regions.

Materials & Methods

Plant sampling

The herbarium specimens analysed in this study are stored in the bryophyte herbarium of the W. Szafer Institute of Botany, Polish Academy of Sciences (KRAM B). The samples used for our tests represented 25 species from 11 families (Table S1). Plant material originated from several austral polar areas, namely: King George Island (South Shetland Islands) in the Antarctic, Marion Island (Prince Edward Islands), Île de la Possession (Îles Crozet) and Îles Kerguelen in the Subantarctic, and from Isla Grande de Tierra del Fuego (southern South America).

Specimens were in the age range from 12 to 39 years with a median of 19. As far as we could reconstruct, all specimens used in this study were air-dried immediately after collection.

Preparation steps

Before sample preparation and DNA extractions, the bench top was cleaned with Fugaten Spray (Medilab, Poland) with one-minute incubation. Forceps were sterilised with ethanol and flamed before each specimen handling. All disposable consumables were DNA-free. Sterile filter tips were used for all experimental procedures. During the preparatory step, whenever possible, green gametophyte vegetative shoots were taken. Considering that large amounts of herbarium voucher material are usually not available, we applied to the presented DNA extraction protocols less than 10 mg of dried tissue, typically around 8 mg. Selected fragments of the dried tissue from herbarium voucher specimens were weighted and disrupted in a mixer mill (MM400 – Qiagen TissueLyser II, Retsch, Germany), using one tungsten bead per sample. Samples were ground two times for 30 seconds at 20 Hz and subsequently used for DNA extraction.

DNA extraction

Total genomic DNA was extracted with four different protocols: column-based DNeasy Plant Mini Kit (Qiagen, Germany), and three variants of CTAB-based extraction method, hereafter referred to as CTAB-ethanol/NaCl[a], CTAB-ethanol/NaCl[b], and CTAB-isopropanol protocols. In the case of the DNeasy Plant Mini Kit, we followed the manufacturer's instruction. CTAB-based protocols have evolved from the extraction methods described by Staats et al. (2011), Särkinen et al. (2012), and Healey et al. (2014), and vary according to the precipitation solution used. A detailed description and major variations of all three CTAB extraction methods used are summarised in Table 1. It should be noted that the number of samples and taxa used in Qiagen test and CTAB extraction tests are different. This is primarily related to the fact, that originally both data were obtained as independent tests performed within different framework. However, the two tests presented provide together a well complementary view on the possible methodological approaches.

DNA quality after extractions was evaluated using two criteria: (1) DNA yield, and (2) PCR amplification success (for a detailed description see below). Here, PCR success was selected as a proxy for evaluating DNA content and purity. The presence of primary and secondary chemicals in plant cells are expected to have inhibiting properties on PCR reaction. Hence, inadequate purification of genomic DNA, especially from polyphenols and polysaccharides, could result in a lack of amplification.

Qiagen extraction test

To verify the general feasibility of obtaining PCR-amplifiable DNA from moss herbarium specimens, DNeasy Plant Mini Kit was selected as a standard approach. This method was selected because most of the recent bryological studies so far relied on this commercially available kit (e.g. Pisa et al., 2014; Wynns & Lange, 2014; Hedenäs, 2014; 2017; Biersma et al., 2017; Biersma et al., 2018a; 2018b).

In this test, we analysed 21 moss species of different age (12-39 years old). Isolation output was tested using PCR amplification of 10 genomic loci of variable length: nuclear ribosomal DNA (5.8*SR*-*ITS2*, *18S*, *adk* and *phy*2), and plastid marker regions (*psbAF*-*trnHR2*, *atpI*-*atpH*, *trnL*-*trnF*, *rps4, atpB1*-*rbcL1*, *psbB*-*clpP*). Genetic studies using herbarium specimens often highlighted the degraded nature of ancient DNA. Hence, when the above PCR tests were negative, we additionally analysed selected short fragments of the plastid *trnS*-*trnF* region.

The total DNA concentration was measured in all samples tested using Invitrogen Qubit 2.0 Fluorometer (Life Technologies, USA) with the Qubit dsDNA High Sensitivity Assay Kit.

CTAB extraction test

In the second step of the tests, we compared the DNeasy Plant Mini Kit based isolation with modified CTAB extraction protocols, less costly and potentially yielding a higher amount of isolated DNA. Here, we used 7 different moss species which were collected over a period of 6-20 years.

To check the quality of the extracted genomic DNA, PCR amplification was performed for genetic regions of the nuclear ribosomal (*ITS5*bryo-*ITSC*bryo, *ITSD*bryo-*ITS4*bryo), and plastid (*trnT*-*trnF*, *rps4*) DNA regions. Within CTAB extraction protocols, the type of precipitation solutions, i.e. ethanol (C_2H_5OH) combined with the sodium chloride (NaCl), and isopropanol (C_3H_8O), as well as the proportions of the ethanol/sodium chloride used in relation to total sample volume, were the key determinants to test the effects on downstream molecular applications. We proposed a modified proportion of ethanol/sodium chloride component (here, protocol CTAB-ethanol/NaCl[b]), differing from the method used by Healey et al. (2014) (here, protocol CTAB-ethanol/NaCl[a]). The modification applied is supposed to increase DNA purity although possibly decreasing DNA concentration. Thus, we have checked whether DNA purity or concentration is more relevant for obtaining PCR-amplifiable DNA from herbarium moss tissue.

CTAB-based methods often provide a weakly purified DNA with contaminants having inhibitory effects on downstream enzymatic treatments, thus we attempted to additionally purify our CTAB extracted samples. To this end, we used the Genomic DNA Clean & Concentrator-10 kit (Zymo Research, USA) according to the manufacturer's recommendation. With this protocol, we used 10 µL of input genomic DNA. Following purification, DNA was eluted from the matrix with 15 µL of the DNA Elution Buffer preheated to 65°C. Usually, the Zymo-Spin matrix absorbs approximately 5 µL volume of the DNA Elution Buffer and the final output was around 10 µL of the purified genomic DNA. In the PCR reactions, we utilized two types of genomic DNA samples, before and after cleaning on the Zymo-Spin matrix, to compare PCR success rate between samples with and without purification. Although silica binding based protocols provide extractions of highly purified DNA samples, Qiagen DNA isolates were additionally purified using Zymo-Spin matrix, in order to allow for a comparison of the final results within this assay.

In this test, Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) an automated on-chip electrophoresis system, was used to evaluate the size distribution of the DNA fragments. For this purpose, the Agilent High Sensitivity DNA kit was selected to provide the optimal

separation of the potentially fragmented DNA. The samples were analyzed following the manufacturer's protocol.

DNA yield was measured using an Invitrogen Qubit 2.0 Fluorometer (Life Technologies, USA) together with the Qubit dsDNA High Sensitivity Assay Kit.

PCR amplification and gel electrophoresis

Based on total DNA isolates, target regions were amplified using the primers listed in Table S2. The PCR of all plastid markers was carried out in accordance to the Shaw et al. (2007) "slow and cold" protocol, whereas nuclear markers were amplified due to the Sabovljević & Frahm (2011) recommendations. In all cases the total volume of PCR mixture was 20 µL and comprised of REDTaq DNA Polymerase (0.05 U/µL) (Sigma-Aldrich, USA), 1x REDTaq Reaction Buffer containing $MgCl_2$ (Sigma-Aldrich, USA), primers forward and reverse (0,2 µM each primer) (Sigma-Aldrich, USA), dNTPs solution (200 µM each dNTP) (Sigma-Aldrich, USA), BSA (0.1 mg/mL) (New England BioLabs, USA), 1 µL template DNA and water. The PCR products were run on a 1% agarose gel stained with SimplySafe (EURx, Poland). All amplicon lengths included in the text are evaluated based on gel electrophoresis (data not shown). We did not use any further improvement of selected PCR protocols, as long as our goal was to check the general feasibility of obtaining PCR-amplifiable DNA after Qiagen and CTAB extractions.

Sequence analysis

Successful amplification products from Qiagen extraction test were treated using enzymatic purification with ExoSAP-IT kit (Affymetrix, USA). We mixed 1 µL of template DNA with 3 µL of ExoSAP-IT solution, and incubated this mixture at 37°C for 15 min, and at 80°C for subsequent 15 min. Cycle sequencing reactions (3 min. 96°C, 30 cycles (10 sec. 96°C, 5 sec. 50°C, 2 min. 60°C)) were carried out in an Mastercycler Nexus thermocyclers (Eppendorf, Germany) using the BigDye Terminator 3.1 chemistry (Thermo Fisher Scientific, USA) along with the BDX64 Enhancing Buffer (MCLAB, USA) in accordance with the manufacturer's protocol for 32x dilution. However, in this case, several duplicate samples were taken to test more fold dilutions, here 64x and 128x. To sum up, all dilutions tested resulted in high-quality data with no significant differences between the obtained sequences. Cycle sequencing reactions were conducted using primers used for PCR. Sequencing reactions were separated in the Applied Biosystems 3130 Series Genetic Analyzer (Thermo Fisher Scientific, USA). The sequence dataset was aligned with Geneious v.10.1.3 (Biomatters Ltd.), using default settings. GenBank accession numbers are listed in Table S3.

Results

Qiagen extraction test

This test included 25 specimens that represented 21 moss species. The PCR and sequencing success for the individual herbarium specimen, and the 10 selected target regions are summarised in Table 2 and Fig. 1. Neither the age nor DNA yield was a good predictor for the successful

PCR reaction. For the sequence lengths, we found that fragment length above 1000 bp strongly affected overall PCR success, in particular in the case of nuclear DNA fragment.

Effect of the DNA yield from herbarium specimens

In our study, the Qiagen extraction method generally yielded low amounts of total DNA with concentration ranging from 0.256 to 8.780 ng/µL (Table 2). Most samples (60%) yielded between 0.200 and 1.950 ng/µL (Table 3). Obtained low yield of DNA template from herbarium samples was not a limiting factor for the successful PCR amplification (Fig. 2). To illustrate, specimens with one of the lowest amount of genomic DNA, i.e range of 0.396 to 0.890 ng/µL, amplified successfully in more than five regions tested (i.e. *Breutelia integrifolia*, *Distichium capillaceum*, *Schistidium halinae*). Meanwhile, specimens with the highest genomic DNA concentration i.e range of 4.000 to 8.780 ng/µL failed to amplify in all target region tested (i.e. *Andreaea nitida*, *Globothecium tortifolium*, *Sanionia uncinata*, *Schistidium* sp.). Also, no amplicon was obtained in some samples where no genomic DNA was detected (i.e. *Notoligotrichum trichodon*, *Polytrichadelphus magellanicus*).

Age of herbarium specimens and PCR success

There was no clear correlation of PCR success with the age of samples (tested in the age range from 12 to 39-year-olds) (Fig. 3). Accordingly, certain of analysed specimens, both from the oldest (i.e. *Sanionia uncinata*), and the youngest (i.e. *Andreaea nitida*, *Frenotia tortifolia*, *Schistidium* sp.) collections did not amplify in any of the selected genetic regions. On the other hand, several of the oldest samples studied (i.e. *Sanionia georgicouncinata*, *Schistidium falcatum*, *Schistidium halinae*, *Warnstorfia fontinaliopsis*) along with the youngest (i.e. *Breutelia integrifolia*, *Distichium capillaceum*, *Ditrichum strictum*, *Racomitrium lanuginosum*, *Sanionia uncinata*) amplified successfully in more than or equal to 5 regions tested, including both nuclear and chloroplast regions.

Locus length and the PCR and sequencing success

After PCR optimization, it was possible to amplify five or more of the target regions in 12 out of 25 studied specimens. A total of 80 sequences (product sequencing in two directions) were obtained from an assay of 10 DNA loci. Four of the target regions were the most difficult to amplify, with PCR success rates in the range of 16-24% for the nuclear *18S*, *adk*, *phy2* genes, and 40% for the chloroplast *psbB-clpP* region. These markers are the longest target regions selected for this study (all region length ca. 1000 bp). In the order of PCR success, the following genetic regions can be specified: nuclear *5.8SR-ITS2* (68%; ca. 450 bp), and plastid *psbAF-trnHR2* (68%; ca. 250 bp), *trnL-trnF* (60%; ca. 450 bp), *atpB1-rbcL1* (52%; ca. 650 bp), *rps4* (48%; ca. 650 bp), and *atpI-atpH* (44%; ca. 550 bp) (see Fig. 1).

With regard to success of bidirectional product sequencing from obtained amplicons, it amounts to: 0% for *18S* and *adk* gene, 30% for *psbB-clpP*, 67% for *phy2* gene, 87% for *trnF-trnL*, 91% for *atpI-atpH*, 92% for *rps4* gene and *atpB1-rbcL1*, and 100% for *psbA-trnH*. The quality of DNA sequences was mostly high and ranged from ca. 250 bp for *psbA-trnH* to ca.

1000 bp for *psbB-clpP* regions. The good quality single-strand data were also obtained in a few cases, and they are marked in Table 2 with an asterisk.

Despite short fragments were targeted, using selected fragments of *trnS-trnF* region, samples that failed into PCR reaction previously (i.e. *Andreaea depressinervis*, *A. nitida*, *Sanionia uncinata*, *Schistidium sp*.) still did not amplify.

CTAB extraction test

The efficiency of DNA extraction using CTAB-ethanol/NaCl[a], CTAB-ethanol/NaCl[b], CTAB-isopropanol, and Qiagen procedures are compared according to PCR success (Fig. 4). Four DNA fragments (nuclear *ITS5*bryo-*ITSC*bryo, *ITSD*bryo-*ITS4*bryo, and plastid *trnT-trnF*, *rps4*), were amplified before and after additional purification using Zymo-Spin matrix. The comparison of four extraction methods showed differences in the number of successfully amplified target regions. The extraction methods of CTAB-ethanol/NaCl[b] with a modified proportion of ethanol/NaCl components and Qiagen had the best overall PCR success. However, when we compared CTAB-ethanol/NaCl[b], and Qiagen extraction, the method that yielded the most amplifiable DNA before purification was CTAB-ethanol/NaCl[b], whereas, after purification treatment, the best performance showed Qiagen extraction. Remaining tested protocols, CTAB-ethanol/NaCl[a], and CTAB-isopropanol had significantly worse performance, in particular before additional DNA cleaning.

We found that additional purification and concentration using Zymo-spin matrix significantly improved the PCR output in all DNA extractions methods tested. The highest PCR success increase was reported in the CTAB-isopropanol method (an increase of 40,6%). For the remaining DNA extractions, the success of PCR amplification after Zymo-spin matrix purification was increased by 28,2% for the Qiagen, 21,9% for the CTAB-ethanol/NaCl[a], and 9,4% for the CTAB-ethanol/NaCl[b] procedure. Furthermore, an improvement of the genomic DNA concentration was observed after using Zymo-Spin kit through all extraction methods tested (Table 4).

The length of the target region appeared to strongly influence the amplification success. Accordingly, the *trnT-trnF* locus was the longest (ca.1500 bp), and the most difficult to amplify. Nevertheless, the Qiagen extraction method in combination with Genomic DNA Clean & Concetrator-10 kit has proved to be the most effective for the successful amplification of the abovementioned genetic region. Remaining loci (*rps4* ca. 600 bp; *ITSD*bryo-*ITS4*bryo ca. 450 bp; *ITS5*bryo-*ITSC*bryo ca. 380 bp) were comparable with respect to PCR success with a small advantage for nuclear *ITS* regions.

The electropherograms obtained by automatic fragment sizing within all extraction methods showed a broad distribution of bands which has indicated that genomic DNA was highly fragmented (Fig. 5, 6, 7). The average sizing of DNA isolate did not vary significantly across methods and is ranged from ca. 400 to 500 bp. Despite the fragment size distribution in all electropherograms remains comparable, the most similar shape of the genomic DNA profiles can be observed among CTAB-ethanol/NaCl[b], and Qiagen extraction method, which could be also reflected in comparable PCR success rate within these two assays.

Discussion

DNA purity rather than concentration as a key factor

Our comparisons highlight the key importance of DNA purity after isolation from herbarium sample, rather than DNA quantity, for successful PCR. Thus, particular attention should be paid to separating DNA from naturally occurring plant cell contaminants, rather than strenuous efforts to obtain high DNA quantity. Among protocols tested in our study, the CTAB-based DNA extraction method provides such a solution, making it a superior choice relative to silica gel column-based commercial kits for DNA extraction.

In the CTAB extraction protocols applied, the performance of the ethanol/NaCl mixture has proven to be crucial for obtaining pure DNA. More precisely, decreasing the volume of the ethanol/NaCl solution to the total volume of extracted sample, i.e. CTAB-ethanol/NaCl^{b} protocol, caused a significant increase of PCR success as opposed to the original proportions applied by Healey et al. (2014). In turn, the proportion of the ethanol/NaCl ingredients has remained unchanged in both variants of the CTAB-ethanol/NaCl based protocols. Likely, reducing the volume of ethanol in our original CTAB protocol may have resulted in a reduced amount of precipitated genomic DNA but in the same time in a significantly lowered concentration of the co-precipitated PCR inhibitors, such as polysaccharides, phenols, and other organic compounds. In general, the addition of a high salt buffer (here, NaCl) could increase genomic DNA purity by a boost of polysaccharides solubility in ethanol, allowing their removal when DNA is pelleted under centrifugation step.

In our CTAB-ethanol/NaCl^{b} extraction protocol, the measured concentration values were the lowest across the tested protocols. It is assumed that DNA yield is good enough to obtain acceptable PCR products if ranged between 6.0—100 ng/µl (Do & Drábková, 2017). In our study, we obtained successful PCR reactions from samples with concentration values lower than 1 ng/µl. Nevertheless, the best performing DNA protocols should be aimed to obtain high purity combined with high DNA yield, which is particularly important in respect of high-throughput sequencing methods. In CTAB extraction tests, the Genomic DNA Clean & Concentrator-10 kit was additionally applied to all prepared DNA extracts. This kit is expected to provide ultra-pure, high-yield genomic DNA. Accordingly, DNA concentration and percentage of the successfully amplified samples has risen significantly after Zymo-Spin cleaning. The main increase in PCR success was observed in the case of the most potentially contaminated extracts, which derived in this study from CTAB-isopropanol, and CTAB-ethanol/NaCl^{a} protocols.

Our results are congruent with several studies which concluded that DNA purity is more important for amplification success than DNA yield (e.g. Höss & Pääbo, 1993; Hänni et al., 1995; Kalmár et al., 2000; Rohland & Hofreiter, 2007; Särkinen et al., 2012. It is worth emphasizing, that plant material could be especially prone to PCR inhibition compared to other organisms. Several different chemical constituents have been found in bryophytes so far (Klavina et al., 2012, Klavina, 2015). Interestingly, Sabovljević, Bijelović &Dragoljub (2001) described bryophytes as “remarkable reservoir” of natural products and/or secondary compounds

such as terpenoids, phenols, glycosides, fatty acids and rare aromatic ingredients. This is also confirmed by Soni & Kumar (2009) who underlined that extraction of DNA from bryophytes could be very difficult due to the presence of secondary compounds inhibiting downstream applications.

Effects of target amplicon size and specimen age on successful PCR

In our tests of extraction protocols, the length of the selected target regions was correlated with the PCR amplification success. This appears an obvious tendency for highly degraded genomic material and our results are in agreement with Särkinen et al. (2012) and Do & Drábková (2017) who indicated that the most easily amplifiable DNA fragments from herbarium material were those below 500 bp. In our report, based on Qiagen extraction test, the best-performing locus are *psbA-trnH* (ca. 250 bp), *trnL-trnF* (ca. 450 bp), and *5.8SR-ITS2* (ca. 450 bp). The most pronounced decrease in PCR success was observed in amplicons around 1000 bp (*18S*, *adk*, *phy2*, *psbB-clpP*, including *trnT-trnF* region from CTAB extraction test), and was more evident in nuclear regions. However, it was possible in some cases to amplify target genomic regions of up to 1500 bp. Thus, even though amplification success declines with target amplicon size for herbarium-based isolates, some collections may provide DNA quality high enough to provide adequate data for molecular analyses. Since short fragments prevail in herbarium DNA, it is expected that PCR of smaller regions has a higher success rate. It is, admittedly, under abovementioned observations, but on the other hand, an attempt to amplify short, barcode regions using samples which failed previously in PCR reaction (within Qiagen extraction test) was still unsuccessful. In a case like this, DNA un-purity may play a more significant role in inhibiting PCR reaction than DNA fragmentation. Possibly, in this particular case, PCR optimisation, using both fresh and herbarium material may result in improvement of successful amplification.

Although in the Qiagen extraction test we did not have an equal share of specimens for a given age, we found no correlation for the age of specimen and PCR success in the age range examined. Successful amplification rate was comparable for the oldest (39 years old) and youngest specimens (12) and rather other factors affecting the collection history seem decisive. Previous studies have also shown that age of herbarium samples had no significant effect on PCR success, pointing out the importance of locus types to be amplified rather than the age (Särkinen et al., 2012; Do & Drábková, 2017). Summarizing, the age of moss specimen should not deter bryologists from their usage in molecular research although certainly at the sample age much exceeding those tested here the impact of DNA fragmentation may gradually appear preponderant.

The rate of DNA degradation in moss herbarium material – CTAB test

Our extraction tests also took into consideration the level of DNA fragmentation in moss herbarium samples. The overall strand breaks of DNA retrieved from the selected moss herbarium specimens was high and only slightly varied between applied extraction methods, and

specimens. However, based on obtained electropherograms and virtual gel imaging output it is possible to notice that the quality of genomic DNA was the most similar for the Qiagen and CTAB-ethanol/NaCl[b] extraction methods. Likely, the comparable level of PCR success obtained based on these two methods can be largely attributed to this and suggests that our modified CTAB-based protocol could offer high-quality DNA from herbarium moss collections, which could correspond to results obtained with Qiagen protocol. We also found no ample difference between obtained DNA profiles for all samples tested, representing age range between 5 to 19 years. Although Weiß et al. (2016) documented the correlation of DNA degradation through time, our samples did not show any age-related fragmentation in the time frame tested. On the other hand, it has been suggested that most DNA fragmentation in herbarium samples occurs on specimen preparation by applying sample drying using a high temperature (60ºC) or alcohol (Staats et al., 2011; Särkinen et al., 2012). Consequently, in our tests, most DNA damage could likely be attributed to sampling method preparation rather than the direct impact of collection age, although the number of specimens used in this test is too small to draw firm conclusions about this aspect. Certainly, in any case, it is important to underline the need for collecting and gathering DNA-friendly material accompanying herbarium collections during expeditions. This could be mostly obtained by using silica gel drying, fixing in appropriate collection buffers or Whatman FTA card technology, as emphasized by Gaudeul & Rouhan (2013).

Conclusions

Our report is the first to offer a ready-to-use CTAB-based DNA extraction protocol tested specifically for moss herbarium specimens. This procedure provides a good alternative to expensive commercial kits, without negatively influencing experiment success. According to our tests, the quality and quantity of DNA obtained with this method is high enough for downstream PCR-based genetic analysis. Our observations regarding factors which influence the usage of moss herbarium material for DNA isolation are congruent with previous studies based on other groups of organisms. DNA purity and targeted amplicon size are more correlated with PCR success than DNA yield. We also showed that examined genomic DNA was highly fragmented, as typical for collection material, but degradation was not correlated with collection age.

Our observations were tested on an array of moss herbarium materials including a large taxon sampling and collection age. Thus, methodological conclusions could be directly adaptable to various molecular studies on mosses based on herbarium material. This seems of special value when taking into account that mosses are main elements of flora in many geographical areas difficult to reach due to field work logistics constraints. Antarctica and the austral polar region, in general, can serve as the prominent example. In such cases, the possibility to efficiently include herbarium specimens in investigation appears of key importance.

Acknowledgments

Sincere thanks are due to Michał Ronikier, and Ryszard Ochyra for critically reading the manuscript and they encouraging support. I am grateful to Ryszard Ochyra for the providing of herbarium specimens. Especially thanks to Michał Ronikier for financial support.

References

Biersma EM, Jackson JA, Bracegirdle TJ, Griffiths H, Linse K, Convey P. 2018a. Low genetic variation between South American and Antarctic populations of the bank-forming moss Chorisodontium aciphyllum (Dicranaceae). *Polar Biology* 41(4): 599-610.

Biersma EM, Jackson JA, Hyvönen J, Koskinen S, Linse K, Griffiths H, Convey P. 2017. Global biogeographic patterns in bipolar moss species. *Royal Society open science* 4(7): 170147.

Biersma EM, Jackson JA, Stech M, Griffiths H, Linse K, Convey P. 2018b. Molecular data suggest long-term in situ Antarctic persistence within Antarctica's most speciose plant genus, Schistidium. *Frontiers in Ecology and Evolution* 6: 77.

Blattner FR. 1999. Direct amplification of the entire ITS region from poorly preserved plant material using recombinant PCR. *BioTechniques* 27(6): 1180-1186.

Cannone N, Seppelt R. 2008. A preliminary floristic classification of southern and northern Victoria Land vegetation, continental Antarctica. *Antarctic Science* 20(6): 553-562.

Cannone N, Convey P, Guglielmin M. 2013. Diversity trends of bryophytes in continental Antarctica. *Polar biology* 36(2): 259-271.

Chiang TY, Schaal BA, Peng CI. 1998. Universal primers for amplification and sequencing a noncoding spacer between the atpB and rbcL genes of chloroplast DNA. *Botanical Bulletin of Academia Sinica* 39.

Convey P, Lewis Smith RI. 2006. Geothermal bryophyte habitats in the South Sandwich Islands, maritime Antarctic. *Journal of Vegetation Science* 17(4): 529-538.

Cota-Sánchez JH, Remarchuk K, Ubayasena K. 2006. Ready-to-use DNA extracted with a CTAB method adapted for herbarium specimens and mucilaginous plant tissue. *Plant Molecular Biology Reporter* 24(2): 161.

Cox CJ, Goffinet B, Newton AE, Shaw AJ, Hedderson TA. 2000. Phylogenetic relationships among the diplolepideous-alternate mosses (Bryidae) inferred from nuclear and chloroplast DNA sequences. *Bryologist* 224-241.

De Castro O, Menale B. 2004. PCR amplification of Michele Tenore's historical specimens and facility to utilize an alternative approach to resolve taxonomic problems. *Taxon* 53(1): 147-151.

de Freitas K, Metz G, Cañon E, Roesch L, Pereira A, Victoria F. 2018. Characterization and Phylogenetic Analysis of Chloroplast and Mitochondria Genomes from the Antarctic Polytrichaceae Species Polytrichum juniperinum and Polytrichum strictum. *Diversity* 10(3): 89.

Do D, Drábková LZ. 2018. Herbarium tale: the utility of dry specimens for DNA barcoding Juncaceae. *Plant Systematics and Evolution* 304(2): 281-294.

Drábková L, Kirschner JAN, Vlček Č. 2002. Comparison of seven DNA extraction and amplification protocols in historical herbarium specimens of Juncaceae. *Plant Molecular Biology Reporter* 20(2): 161-175.

Fraser CI, Connell L, Lee CK, Cary SC. 2018. Evidence of plant and animal communities at exposed and subglacial (cave) geothermal sites in Antarctica. *Polar Biology* 41(3): 417-421.

Fraser CI, Terauds A, Smellie J, Convey P, Chown SL. 2014. Geothermal activity helps life survive glacial cycles. *Proceedings of the National Academy of Sciences* 201321437.

Gaudeul M, Rouhan G. 2013. A plea for modern botanical collections to include DNA-friendly material. *Trends in Plant Science* 18(4): 184-185.

Goff LJ, Moon DA. 1993. PCR amplification of nuclear and plastid genes from algal herbarium specimens and algal spores. *Journal of Phycology* 29(3): 381-384.

Hänni C, Brousseau T, Laudet V, Stehelin D. 1995. Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. *Nucleic acids research* 23(5): 881.

Healey A, Furtado A, Cooper T, Henry RJ. 2014. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant methods* 10(1): 21.

Hedenäs L. 2014. Intraspecific genetic variation in selected mosses of Scandinavian interglacial refugia suggests contrasting distribution history patterns. *Botanical journal of the Linnean Society* 176(3): 295-310.

Hedenäs L. 2017. Three molecular markers suggest different relationships among three Drepanocladus species (Bryophyta: Amblystegiaceae). *Plant Systematics and Evolution* 303(4): 521-529.

Hills SF, Stevens MI, Gemmill CE. 2010. Molecular support for Pleistocene persistence of the continental Antarctic moss Bryum argenteum. *Antarctic Science* 22(6): 721-726.

Höpke J, Brewer G, Dodsworth S, Ortiz EM, Albach DC. 2019. DNA extraction from old herbarium material of Veronica subgen. Pseudolysimachium (Plantaginaceae). *Ukrainian Botanical Journal* 75(6): 564-575.

Hopple JrJS, Vilgalys R. 1999. Phylogenetic relationships in the mushroom genus Coprinus and dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups, and monophyly. *Molecular phylogenetics and evolution* 13(1): 1-19.

Höss M, Pääbo S. 1993. DNA extraction from Pleistocene bones by a silica-based purification method. *Nucleic acids research* 21(16): 3913.

Jankowiak K, Buczkowska K, Szweykowska-Kulinska Z. 2005. Successful extraction of DNA from 100-year-old herbarium specimens of the liverwort Bazzania trilobata. *Taxon* 54(2): 335-336.

Jiménez JA, Ochyra R. 2017. Reinstatment of species rank for Didymodon gelidus (Bryophyta, Pottiaceae). *Cryptogamie, Bryologie* 38(4): 383-393.

Kalmár T, Bachrati CZ, Marcsik A, Raskó I. 2000. A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. *Nucleic Acids Research* 28(12): e67-e67.

Kato K, Arikawa T, Imura S, Kanda H. 2013. Molecular identification and phylogeny of an aquatic moss species in Antarctic lakes. *Polar biology* 36(11): 1557-1568.

Klavina L. 2015. Polysaccharides from lower plants: bryophytes. *Polysaccharides: Bioactivity and Biotechnology* 145-160.

Kļaviņa L, Bikovens O, Šteinberga I, Maksimova V, Eglīte L. 2012. Characterization of chemical composition of some bryophytes common in Latvia. *Environmental and Experimental Biology* 10: 27-34.

Lavoie C. 2013. Biological collections in an ever changing world: Herbaria as tools for biogeographical and environmental studies. *Perspectives in Plant Ecology, Evolution and Systematics* 15(1): 68-76.

Lehtonen S, Christenhusz MJ. 2010. Historical herbarium specimens in plant molecular systematic - an example from the fern genus Lindsaea (Lindsaeaceae). *Biologia* 65(2): 204-208.

Li SP, Ochyra R, Wu PC, Seppelt RD, Cai MH, Wang HY, Li CS. 2009. Drepanocladus longifolius (Amblystegiaceae), an addition to the moss flora of King George Island, South Shetland Islands, with a review of Antarctic benthic mosses. *Polar Biology* 32(10): 1415-1425.

Marko S, Aneta B, Dragoljub G. 2001. Bryophytes as a potential source of medicinal compounds. *Pregledni članak - Review* 21(1): 17-29.

McDaniel SF, Shaw AJ. 2005. Selective sweeps and intercontinental migration in the cosmopolitan moss Ceratodon purpureus (Hedw.) Brid. *Molecular Ecology* 14(4): 1121-1132.

Ochyra R, Bednarek-Ochyra H, Lewis Smith RI. 2008. New and rare moss species from the Antarctic. *Nova Hedwigia* 87(3-4), 457-477.

Ochyra R, Lewis Smith RI, Bednarek-Ochyra H. 2008. *The illustrated moss flora of Antarctica*. Cambridge: Cambridge University Press.

Peat HJ, Clarke A, Convey P. 2007. Diversity and biogeography of the Antarctic flora. *Journal of Biogeography* 34(1): 132-146.

Piñeiro R, Popp M, Hassel K, Listl D, Westergaard KB, Flatberg KI, Stenøien HK, Brochmann C. 2012. Circumarctic dispersal and long-distance colonization of South America: the moss genus Cinclidium. *Journal of Biogeography* 39(11): 2041-2051.

Pisa S, Biersma EM, Convey P, Patiño J, Vanderpoorten A, Werner O, Ros RM. 2014. The cosmopolitan moss Bryum argenteum in Antarctica: recent colonisation or in situ survival? *Polar biology* 37(10): 1469-1477.

Porebski S, Bailey LG, Baum BR. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant molecular biology reporter* 15(1): 8-15.

Quandt D, Stech M. 2004. Molecular evolution of the trnTUGU-trnFGAA region in bryophytes. *Plant biology* 6(5): 545-554.

Rankin AH, Pressel S, Duckett J, Rimington WR, Hawes I, Sumner DY, Mackey TJ, Castendyke D, Schneider H, Jungblut AD. 2017. Characterisation of a deep-water moss from the perennially ice-covered Lake Vanda, Antarctica. *Polar Biology* 40(10): 2063-2076.

Rogers SO, Bendich AJ. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant molecular biology* 5(2): 69-76.

Rohland N, Hofreiter M. 2007. Ancient DNA extraction from bones and teeth. *Nature protocols* 2(7): 1756.

Ronikier M, Saługa M, Jiménez JA, Ochyra R, Stryjak-Bogacka M. 2018. Multilocus DNA analysis supports Didymodon gelidus (Musci, Pottiaceae) as a distinct endemic of the austral polar region. *Acta Societatis Botanicorum Poloniae* 87(4).

Sabovljević M, Bijelović A, Dragoljub G. 2001. Bryophytes as a potential source of medicinal compounds. *Pregledni članak - Review* 21(1): 17-29.

Sabovljević M, Frahm JP. 2011. Genetic diversity of the relict moss Rhytidium rugosum (Hypnales) in Europe inferred from the ITS region (nrDNA). *Biologia* 66(1): 42-49.

Saługa M, Ochyra R, Żarnowiec J, Ronikier M. 2018. Do Antarctic populations represent local or widespread phylogenetic and ecological lineages? Complicated fate of bipolar moss concepts with Drepanocladus longifolius as a case study. *Organisms Diversity & Evolution* 18(3): 263-278.

Särkinen T, Staats M, Richardson JE, Cowan RS, Bakker FT. 2012. How to open the treasure chest? Optimising DNA extraction from herbarium specimens. *PloS one* 7(8): e43808.

Savolainen V, Cuénoud P, Spichiger R, Martinez MD, Crèvecoeur M, Manen JF. 1995. The use of herbarium specimens in DNA phylogenetics: evaluation and improvement. *Plant Systematics and Evolution* 197(1-4): 87-98.

Shaw J, Lickey EB, Schilling EE, Small RL. 2007. Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American journal of botany* 94(3): 275-288.

Shepherd L, Perrie L. 2014. Genetic analyses of herbarium material: Is more care required? *Taxon* 63(5): 972-973.

Shepherd LD. 2017. A non-destructive DNA sampling technique for herbarium specimens. *PloS one* 12(8): e0183555.

Sollman P. 2015. The genus Bryoerythrophyllum (Musci, Pottiaceae) in Antarctica. *Polish botanical journal* 60(1): 19-25.

Soni A, Kumar A. 2009. Protocol for improved extraction and PCR amplification of genomic DNA from liverwort, Plagiochasma appendiculatum. *Indian Journal of Experimental Biology* 47: 921-924.

Souza-Chies TT, Bittar G, Nadot S, Carter L, Besin E, Lejeune B. 1997. Phylogenetic analysis ofIridaceae with parsimony and distance methods using the plastid gene rps4. *Plant Systematics and Evolution* 204(1-2): 109-123.

Staats M, Cuenca A, Richardson JE, Vrielink-van Ginkel R, Petersen G, Seberg O, Bakker F. T. 2011. DNA damage in plant herbarium tissue. *PLoS One* 6(12): e28448.

Staats M, Erkens RH, van de Vossenberg B, Wieringa JJ, Kraaijeveld K, Stielow B, Geml J, Richardson JE, Bakker FT. 2013. Genomic treasure troves: complete genome sequencing of herbarium and insect museum specimens. *PLoS One* 8(7): e69189.

Stech M, Frey W. 2008. A morpho-molecular classification of the mosses (Bryophyta). *Nova Hedwigia* 86(1-2): 1-21.

Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T, Corthier G, Brochmann CH, Willerslev E. 2006. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic acids research* 35(3): e14-e14.

Telle S, Thines M. 2008. Amplification of cox2 (~ 620 bp) from 2 mg of up to 129 years old herbarium specimens, comparing 19 extraction methods and 15 polymerases. *PLoS One* 3(10): e3584.

Van der Putten N, Verbruggen C, Ochyra R, Spassov S, De Beaulieu JL, De Dapper M, Hus J, Thouveny N. 2009. Peat bank growth, Holocene palaeoecology and climate history of South Georgia (sub-Antarctica), based on a botanical macrofossil record. *Quaternary Science Reviews* 28(1-2): 65-79.

649 Waterman MJ, Nugraha AS, Hendra R, Ball GE, Robinson SA, Keller PA. 2017. Antarctic moss
650 biflavonoids show high antioxidant and ultraviolet-screening activity. *Journal of natural*
651 *products* 80(8): 2224-2231.
652
653 Weiß CL, Schuenemann VJ, Devos J, Shirsekar G, Reiter E, Gould BA, Stinchcombe JR, Krause
654 J, Burbano HA. 2016. Temporal patterns of damage and decay kinetics of DNA retrieved from
655 plant herbarium specimens. *Royal Society open science* 3(6): 160239.
656
657 White TJ, Bruns T, Lee S, Taylor JL. 1990. Amplification and direct sequencing of fungal
658 ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and*
659 *applications* 18(1): 315-322.
660
661 Wynns JT, Lange CBA. 2014. A comparison of 16 DNA regions for use as phylogenetic markers
662 in the pleurocarpous moss genus Plagiothecium (Hypnales). *American journal of botany* 101(4):
663 652-669.

Table 1(on next page)

Table 1: CTAB extraction test. DNA extraction protocol.

1

Add extraction buffer		
Add to the each sample 1mL of preheated to 65^{0}C 2x CTAB buffer containing β-mercapthoethanol.		
Preparation of 2x CTAB buffer (250 mL): 25 mL (1M) Tris-HCl pH 7,5 + 75 mL (5M) NaCl + 12,5 mL (0,5M) EDTA + 5g CTAB + water until 250 ml Final concentration: (100mM) Tris-HCl + (1,5M) NaCl + (25mM) EDTA + (2%) CTAB (w/v) Add immediately just before use: β-mercapthoethanol 0,3% (v/v) — 5 µL/1000 µL solution		
Incubate the sample at 65^{0}C for 60 min with mixing by inversion every 10 min.		
Centrifuge at 5,000 rcf for 5 min to pellet and remove un-lysed leaf tissue. Transfer the extract to a new 2 mL tubes.		
Protein extraction and RNAse treatment		
Add an equal volume of chloroform : isoamyl alcohol (24:1) to the extract and mix gently. Extract for 30 min by rocking on orbital shaker.		
Centrifuge at 13,000 rpm for 10 min.		
Transfer the upper phase (containing DNA) to a new 2 mL tubes. Take care to avoid the aqueous/organic layer interface.		
Add 1 µL of RNase A solution (10 mg/mL) per 100 µL DNA solution and incubate at 37^{0}C for 15 min with periodic, gentle mixing.		
Repeat the chloroform : isoamyl alcohol extraction to clear the aqueous phase.		
Precipitation		
Add **X** volume of 5M NaCl to the transferred aqueous phase and mix gently by inversion. Then add **Y** volume(s) of pre-chilled (-20^{0}C) 95% ethanol and mix gently by inversion. Incubate at -20^{0}C for 60 min. Note: do not leave the sample at -20^{0}C for more than 60 min as both the CTAB and NaCl can precipitate from solution, preventing DNA isolation.		Add 1,8 volume of pre-chilled (-20^{0}C) isopropanol to the transferred aqueous phase and mix gently by inversion. Incubate at -20^{0}C for 24h.
CTAB-ethanol/ NaCl[a] **X** = 0,5 **Y** = 3 Healey et al., 2014	CTAB-ethanol/NaCl[b] **X** = 0,1 **Y** = 0,6 our modification **Attention** DNA pellets are poorly visible.	CTAB-isopropanol
Centrifuge at 14,000 rpm for 20 min to collect precipitate, Pour off the liquid and add 750 µL of pre-chilled (-20^{0}C) 70% ethanol, Spin down DNA at 13,000 rpm for 15 min, Pour off the liquid and air-dry DNA pellet for 15 min – 30 min at room temperature or dry the samples in vacuum centrifuge for 5 min, Note: in case of isopropanol precipitation wash the pellet 5 times with 750 µL pre-chilled (-20^{0}C) 70% ethanol.		
Dissolve in Tris-EDTA buffer (TE buffer) pH 8,0		
Preparation of TE buffer (500 mL): 5 mL (1M) Tris pH 8 + 1 mL (0,5M) EDTA pH 8 + water until 500 mL Resuspend DNA in 80 µL of TE buffer,		

2

Table 2(on next page)

Table 2: Qiagen extraction test. Specimen information and PCR amplification success. Sequences lengths are estimated with the Geneious software after removal low-quality ends.

1

Lp.	Species	Kram B	Origin	gDNA [ng/µl]	Age	*5.8SR ITS2*	*18S*	*adk gene*	*phy2 gene*	*psbAF trnHR2*	*atpH atpI*	*trnL trnF*	*rps4 gene*	*atpB1 rbcL1*	*psbB clpP*
						Coloured background (successfull PCR / sample locus length [bp]*)									
1.	*Andreaea depressinervis*	4928/79	KGI	0.466	39	*									
2.	*A. nitida*	674026	KER	4.060	12										
3.	*Blindia magellanica*	611/99	MAR	1.920	19	458				246	374	468	658	602	
4.	*Brachythecium subplicatum*	614/99	MAR	3.400	19	439	782*		*	256	557	443	648	680	1097
5.	*Breutelia integrifolia*	124/06	POSS	0.890	12	448		*		247	469	468	660	653	637*
6.	*Bucklandiella heterostichoides*	695575	KER	1.630	12	443				244		316*			
7.	*Cratoneuropsis chilensis*	403/99	MAR	1.310	19	389				256	557	420	676	689	676*
8.	*Distichium capillaceum*	1198/06	KER	1.430	12	449		*	714	228	582	511	665	659	1045
9.	*Ditrichum strictum*	194/06	POSS	0.396	12	471	754*		895	244	499	169	665	629	449*
10.	*Hymenoloma antarcticum*	2662/80	KGI	1.720	38	*				246					
11.	—	110401	KGI	0.594	29					251		501		573	
12.	*Hymenoloma tortifolium*	527564	KER	8.780	12										
13.	*Notoligotrichum trichodon*	487/95	FUE	too low	23										
14.	*Polytrichadelphus magellanicus*	302/95	FUE	too low	23										
15.	*Racomitrium lanuginosum*	43266	POSS	3.340	12	*	*	*		238	528	470	660	671	547*
16.	*Sanionia uncinata*	2/06	POSS	1.710	12	437	*			247	563	418	609	676	1078
17.	—	2268/80	KGI	4.000	38										

18.	*S. georgicouncinata*	454/80	KGI	3.800	38	433	774*	693*		255	540	434	655	632	643*
19.	*Schistidium falcatum*	408/95	FUE	0.256	23	394				248		*			
20.	—	437/80	KGI	2.180	38	*		*		246			646	*	
21.	—	1447/99	MAR	1.810	19	*				238	423*	434			
22.	*S. halinae*	2711/80	KGI	0.722	38	396				243		434	*	450	*
23.	*Schistidium species*	44713	KER	4.780	12										
24.	*Valdonia microcarpa*	555/99	MAR	1.310	19										
25.	*Warnstorfia fontinaliopsis*	1193/80	KGI	1.120	38	431	777*	*	*	251	552	417	665	624	548*

Abbreviattion used: FUE (Isla Grande de Tierra del Fuego, southern South America; KER (Îles Kerguelen); KGI (King George Island, South Shetland Islands); MAR (Marion Island, Prince Edward Islands); POSS (Île de la Possesion, Îles Crozet); * single-stranded read or unsuccessful sequencing

Table 3(on next page)

Table 3: Qiagen extraction test. The number of specimens within a designed genomic DNA concentration range.

1
2

DNA yield [ng/µL]	Number of specimens within given range
too low	2
0,20 — 1,95	**15**
1,96 — 3,71	3
3,72 — 5,47	4
5,48 — 7,23	0
7,24 — 8,99	1
Total number of specimens	25

Table 4(on next page)

Table 4: CTAB extraction test. Specimen information and DNA yield measured before and after using Genomic DNA Zymo Clean & Concentrator-10 kit.

Lp.	Species	Kram B	Origin	Age	gDNA [ng/µL]							
					Before Zymo-Spin				After Zymo-Spin			
					CTAB-ethanol/NaCl[a]	CTAB-ethanol/NaCl[b]	CTAB-isopropanol	Qiagen kit	CTAB-ethanol/NaCl[a]	CTAB-ethanol/NaCl[b]	CTAB-isopropanol	Qiagen kit
1.	*Brachythecium rutabulum*	1363/99	MAR	19	2.120	1.920	2.080	2.410	10.800	6.850	10.225	8.112
2.	*Breutelia integrifolia*	3597/06	KER	12	0.920	0.124	1.170	0.314	5.250	2.370	8.550	2.150
3.	*Bucklandiella striatipila*	3758/06	KER	12	0.845	0.025	0.444	0.030	2.500	1.650	2.750	1.956
4.	*Cratoneuropsis chilensis*	403/99	MAR	19	6.985	3.120	6.720	1.640	15.125	10.000	17.120	7.125
5.	*C. chilensis*	1448/99	MAR	19	4.920	4.480	17.000	5.160	15.025	15.500	51.336	22.650
6.	*Holodontium strictum*	3581/06	KER	12	1.680	0.748	2.550	0.700	6.780	3.250	9.656	2.850
7.	*Rhacocarpus purpurascens*	613/13	KER	05	0.656	0.540	1.510	0.256	2.050	2.450	4.885	2.100
8.	*Valdonia microcarpa*	555/99	MAR	19	1.190	1.170	2.660	1.020	4.450	4.200	8.750	3.850

Abbreviattion used: KER (Îles Kerguelen); MAR (Marion Island, Prince Edward Islands)

Figure 1

Figure 1: Qiagen extraction test. PCR success (%) of selected genetic regions used, measured as the number of positive amplicons divided by the total number of samples.

All indicated lengths of target genetic region are evaluated based on gel electrophoresis and marked above the graphs.

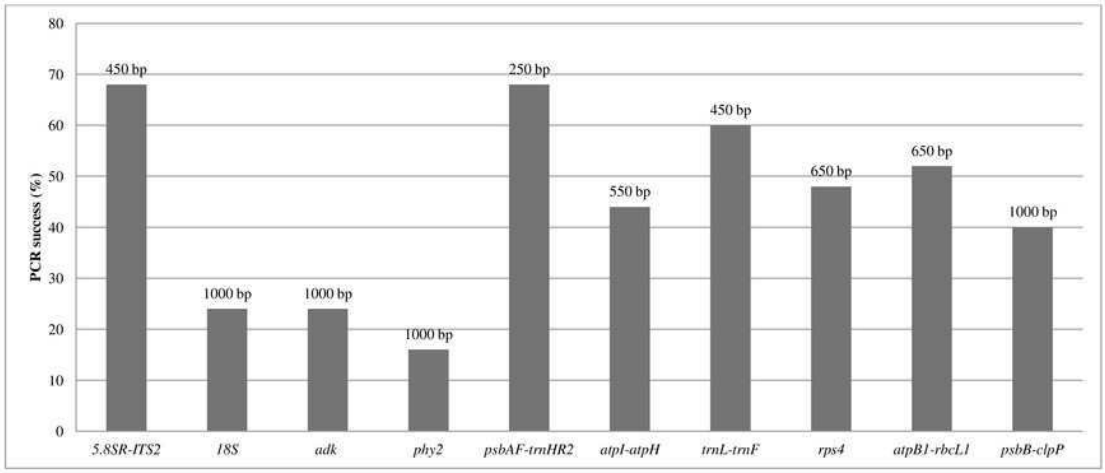
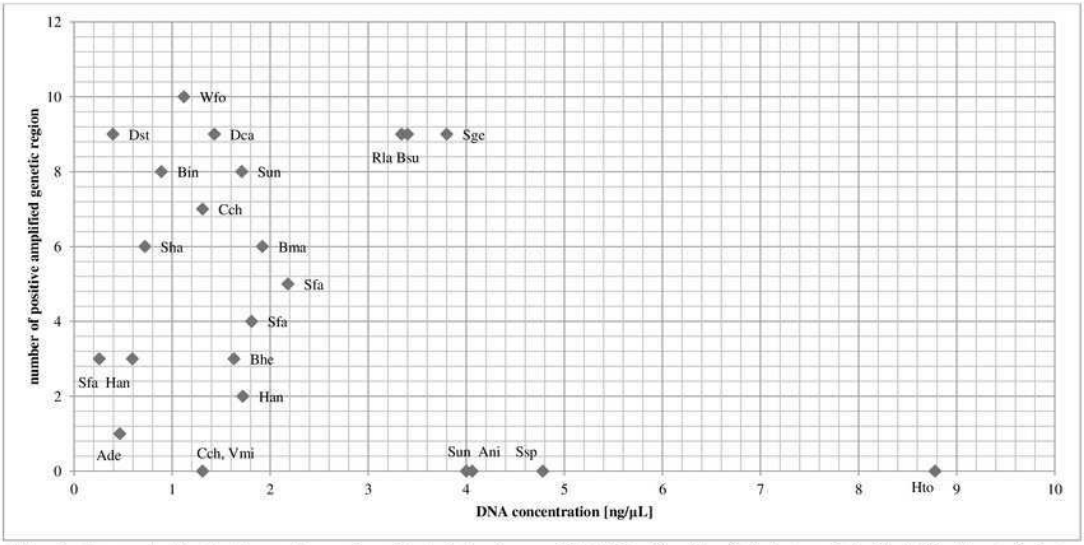


Figure 2

Figure 2: Qiagen extraction test. The impact of DNA concentration values represented by a given moss species on PCR success measured as a number of positive amplified genetic regions.



Abbreviattion used: Ade (*Andreaea depressinervis*); Ani (*Andreaea nitida*); Bhe (*Bucklandiella heterostichoides*); Bin (*Breutelia integrifolia*); Bma (*Blindia magellanica*); Bsu (*Brachythecium subplicatum*); Cch (*Cratoneuropsis chilensis*); Dca (*Distichium capillaceum*); Dst (*Ditrichum strictum*); Han (*Hymenoloma antarcticum*); Hto (*Hymenoloma tortifolium*); Rla (*Racomitrium lanuginosum*); Sfa (*Schistidium falcatum*); Sge (*Sanionia georgicouncinata*); Sha (*Schistidium halinae*); Ssp (*Schistidium* sp.); Sun (*Sanionia uncinata*); Vmi (*Valdonia microcarpa*); Wfo (*Warnstorfia fontinaliopsis*)

Figure 3

Figure 3: Qiagen extraction test. Effect of specimen age on PCR success.

PCR success (%) measured as the number of positive amplified genetic regions in the age range examined divided by the (10*n) where 10, indicates the number of genetic regions tested; n, indicates the number of specimen in given ages (marked above the graphs).

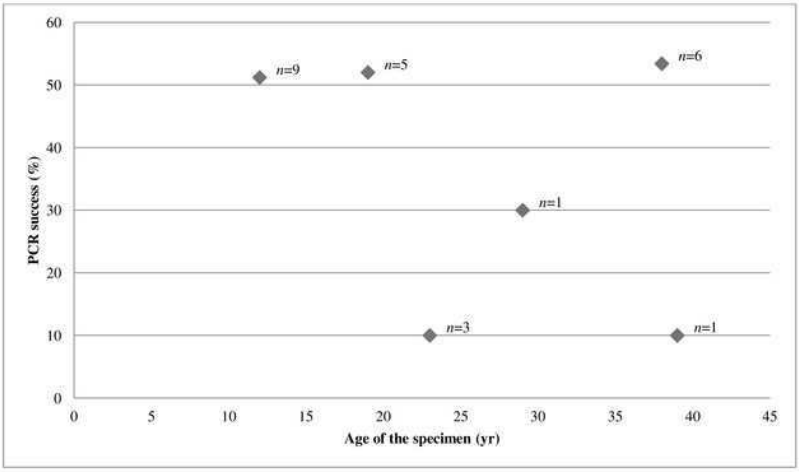


Figure 4

Figure 4: CTAB extraction test. Effect of extraction method on PCR success (%) measured as the number of positive amplicons divided by the total number of samples, before and after using Genomic DNA Clean & Concentrator-10 kit.

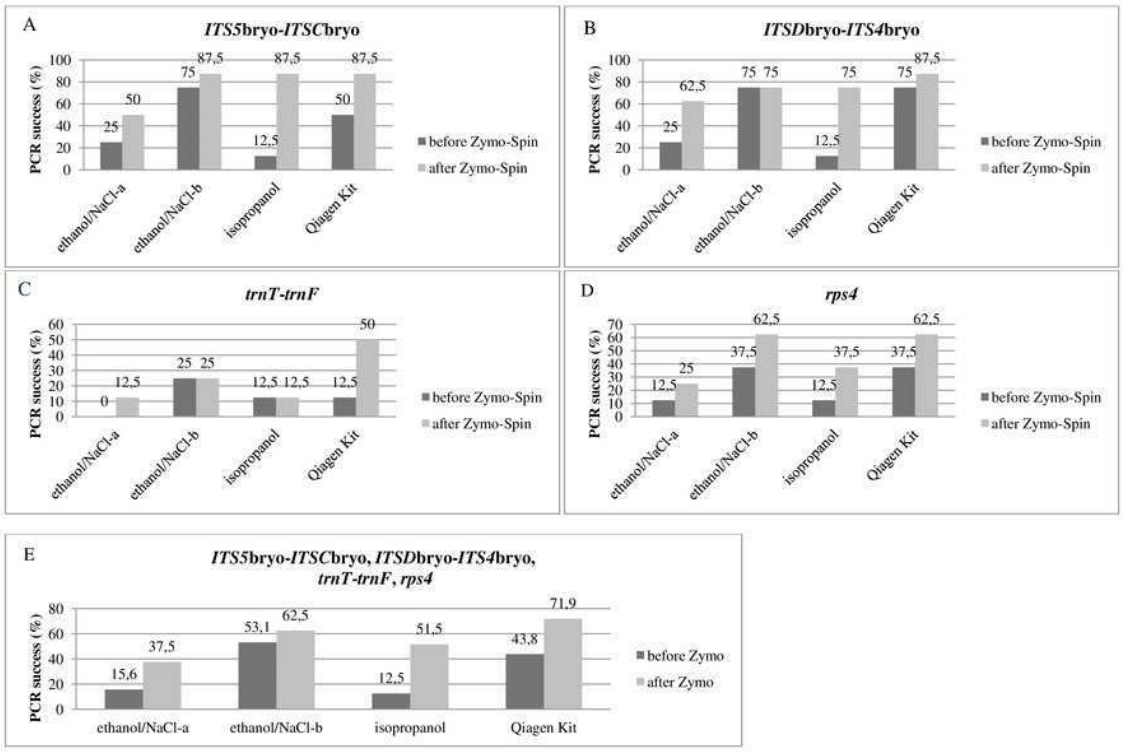


Figure 5

CTAB extraction test. Electropherograms, gel images output (virtual gel) and average fragment size (bp) of genomic DNA analyzed using the Agilent High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer System. Part A

Abbreviation used: [FU]-fluorescense units (DNA amount), [s]-seconds.

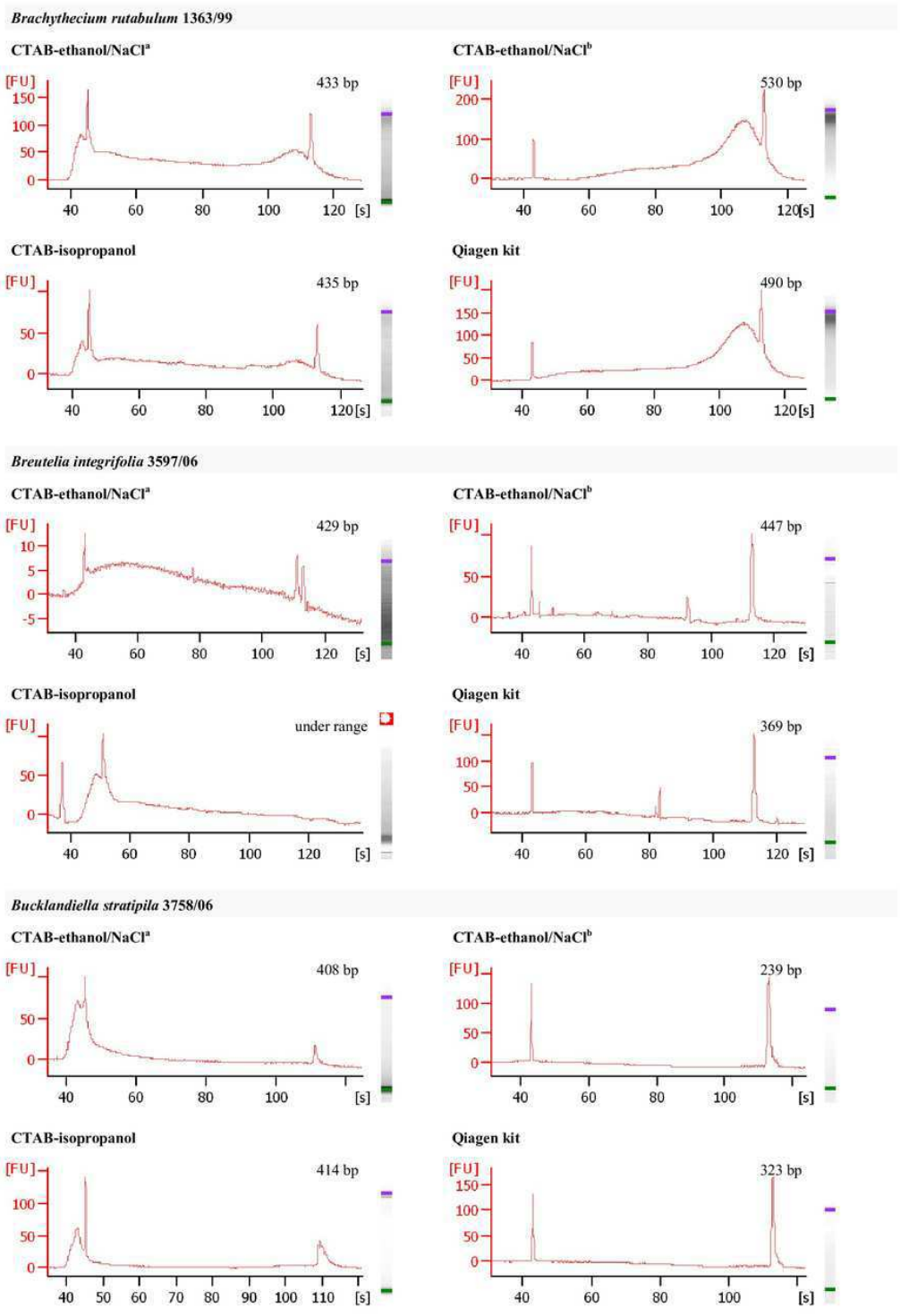


Figure 6

CTAB extraction test. Electropherograms, gel images output (virtual gel) and average fragment size (bp) of genomic DNA analyzed using the Agilent High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer System. Part B

Abbreviation used: [FU]-fluorescense units (DNA amount), [s]-seconds.

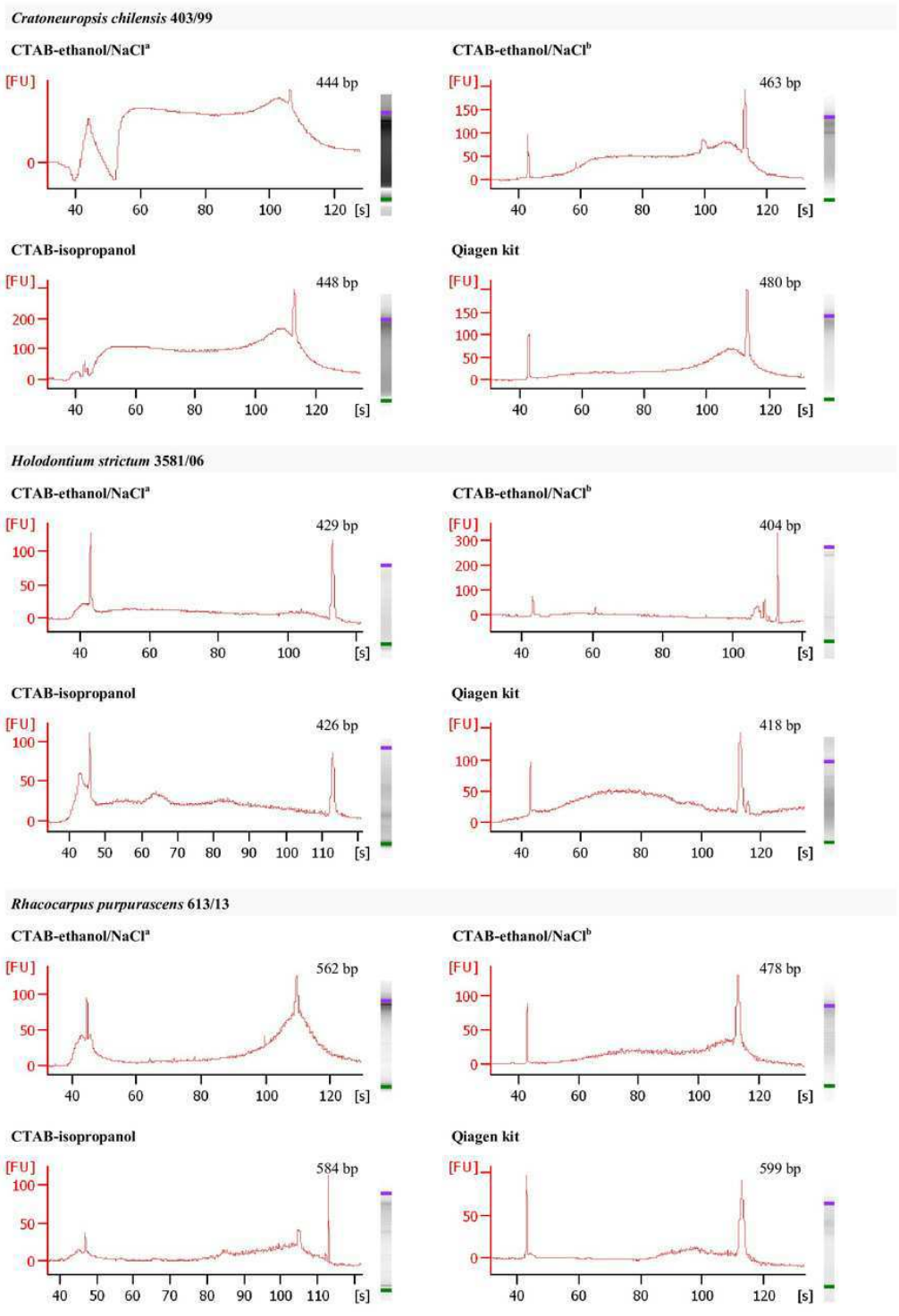


Figure 7

CTAB extraction test. Electropherograms, gel images output (virtual gel) and average fragment size (bp) of genomic DNA analyzed using the Agilent High Sensitivity DNA kit on the Agilent 2100 Bioanalyzer System. Part C

Abbreviation used: [FU]-fluorescense units (DNA amount), [s]-seconds.