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

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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.

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15 **Abstract**

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28 tested and compared the silica column-based method and three variants of CTAB-based DNA
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30 between 1979 and 2013 and specifically focused on austral polar regions to assess the potential
31 of herbarium as a source of biological material from geographical regions of difficult and
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36 successful PCR reaction. Conversely, the specimen age seems to be less relevant. Moreover, the
37 size distribution of the DNA fragments extracted using Qiagen protocol is shown to be
38 comparable to our original CTAB-based approach. Our modified CTAB-based method provides

39   a high-purity genomic DNA allowing efficient downstream amplification. It is not outcompeted
40   by the column-based method and appears as a method of choice in
41   molecular studies of moss herbarium material.
42

Introduction

44   In recent years, assessing techniques of DNA isolation and sequencing from herbarium plant
45   material has gained broad interest (e.g. Rogers & Bendich, 1985; Goff & Moon, 1993;
46   Savolainen et al., 1995; Porebski, Bailey & Baum, 1997; Blattner, 1999; Drábková, Kirschner &
47   Vlĉek, 2002; DeCastro & Menale, 2004; Jankowiak, Buczkowska & Szweykowska-Kulinska,
48   2005; Cota-Sánchez, Remarchuk & Ubayasena, 2006; Telle & Thines, 2008; Soni & Kumar,
49   2009; Lehtonen & Christenhusz, 2010; Staats et al., 2011; Särkinen et al., 2012; Staats et al.,
50   2013; Shepherd & Perrie, 2014; Shepherd, 2017; Do & Drábková, 2018; Höpke et al., 2019).
51   The primary aim of such technical investigations is to establish appropriate methods of efficient
52   DNA analysis from preserved plant specimens, taking into account the specificity of such source
53   material. This specificity includes especially DNA damage in historical material, notable
54   degradation into short fragments as well as chemical modifications which may result in the poor
55   success of genomic DNA isolation and subsequent PCR amplification of DNA fragments (Staats
56   et al., 2011). Accordingly, the success rate of DNA amplification based on fresh samples is
57   substantially higher than that based on herbarium specimens (Shepherd & Perrie, 2014).
58   While DNA analysis from herbarium material presents technical difficulties, sharing
59   empirically tested innovative molecular protocols for biological collections is of major
60   importance (e.g., Lavoie, 2013). Exploration of a wealth of materials deposited in biological
61   museum collections has an invaluable potential for taxonomic and phylogenetic studies but also
62   biogeographical and environmental research. Owing to herbaria sources, there is also an
63   opportunity for collecting large data set including specimens of various taxonomic position,
64   habitats, and geographical distribution patterns, without the need for undertaking field
65   expeditions. Moreover, herbarium collections are of critical importance in cases where adequate
66   taxonomical and/or geographical sampling of fresh material in the field is difficult or impossible.
67   Here, biological collections from hardly accessible regions could especially play an
68   indispensable role.
69   In spite of growing accumulation of published technical experience based on empirical
70   tests, it is important to notice that success of extraction and PCR amplification of herbarium
71   DNA may be strongly affected by factors specific for taxonomical groups, taxa and even
72   specimens (Särkinen et al., 2012). For some taxonomic groups, focused reports of step-by-step
73   improvements of herbarium DNA extraction and amplification methods are still scarce. This is
74   the case of bryophytes, which contrasts with current interest and a central position of molecular
75   studies in moss biology. Collections of mosses deposited in herbaria worldwide may provide an
76   excellent source for DNA based bryological studies.
77   Mosses, being organisms well adapted to many environmental constraints, are often key
78   components of flora in inaccessible and ecologically inhospitable environments. One extreme

79   example is Antarctic biome, locked-in ice continent with only 0.18% to 0.32% of the area being

80   ice-free. Altogether, 115 species of mosses are known from Antarctica (Ochyra, Lewis Smith &

81   Bednarek-Ochyra, 2008; Ochyra, Bednarek-Ochyra & Lewis Smith, 2008; Sollman, 2015;

82   Jiménez & Ochyra, 2017; Ronikier et al., 2018). However, botanical exploration of the Antarctic

83   continent and the maritime Antarctic islands is highly restricted due, among others, to substantial

84   logistic limitations. While herbaria can be very important for studies focused on this area, the

85   number of molecular studies using herbarium specimens from polar regions of the Southern

86   Hemisphere is very low. Lavoie (2013) found only three articles that used herbarium specimens

87   from Antarctica for documenting biogeographical patterns or environmental changes. Since then,

88   we have found only a handful of further studies dealt with herbarium moss material from

89   Antarctica including our recent contributions (Pisa et al., 2014; Biersma et al., 2018a; 2018b;

90   Ronikier et al., 2018; Saługa et al., 2018). On the other hand, there is a clearly growing interest

91   in research focused on mosses from the high-latitude ecosystems of the Southern Hemisphere

92   and related to their biogeography, evolutionary history and ecology (e.g. Convey & Lewis Smith,

93   2006; Peat, Clarke & Convey, 2007; Cannone & Seppelt, 2008; Ochyra, Bednarek-Ochyra &

94   Lewis Smith, 2008; Ochyra, Lewis Smith & Bednarek-Ochyra, 2008; Li et al., 2009; Van der

95   Putten et al., 2009; Hills, Stevens & Gemmill, 2010; Cannone, Convey & Guglielmin, 2013;

96   Kato et al., 2013; Fraser et al., 2014; Pisa et al., 2014; Biersma et al., 2017; Rankin et al., 2017;

97   Waterman et al., 2017; Biersma et al., 2018a; 2018b; Fraser et al., 2018; de Freitas et al., 2018;

98   Ronikier et al., 2018; Saługa et al., 2018).

99   The paper aims to comprehensively examine the methods of improving the use of moss

100   herbarium material for Sanger DNA sequencing. We present a test of DNA isolation methods for

101   herbarium moss specimens and assess their utility. Then, we test the obtained DNA isolates as

102   sources for successful PCR amplification of selected target regions in the length range of no

103   more than 100-150 bp to approximately 1500 bp from low concentrated samples (c. 2,0 ng/μL)

104   of up to 39 years old herbarium specimens. In our report, we consider in particular the age of

105   specimens used, DNA quality and quantity, target amplicon size and DNA severe fragmentation.

106   To estimate a direct relevance for biological studies focused on difficult geographical territories,

107   we deliberately based our analyses on moss materials from the hardly accessible austral polar

108   regions.

Materials & Methods

Plant sampling

111   The herbarium specimens analysed in this study are stored in the bryophyte herbarium of the W.

112   Szafer Institute of Botany, Polish Academy of Sciences (KRAM B). The samples used for our

113   tests represented 25 species from 11 families (Table S1). Plant material originated from several

114   austral polar areas, namely: King George Island (South Shetland Islands) in the Antarctic,

115   Marion Island (Prince Edward Islands), Île de la Possession (Îles Crozet) and Îles Kerguelen in

116   the Subantarctic, and from Isla Grande de Tierra del Fuego (southern South America).

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

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

130 **DNA extraction**
131 Total genomic DNA was extracted with four different protocols: column-based DNeasy Plant
132 Mini Kit (Qiagen, Germany), and three variants of CTAB-based extraction method, hereafter
133 referred to as CTAB-ethanol/NaCl[a], CTAB-ethanol/NaCl[b], and CTAB-isopropanol protocols. In
134 the case of the DNeasy Plant Mini Kit, we followed the manufacturer's instruction. CTAB-based
135 protocols have evolved from the extraction methods described by Staats et al. (2011), Särkinen et
136 al. (2012), and Healey et al. (2014), and vary according to the precipitation solution used. A
137 detailed description and major variations of all three CTAB extraction methods used are
138 summarised in Table 1. It should be noted that the number of samples and taxa used in Qiagen
139 test and CTAB extraction tests are different. This is primarily related to the fact, that originally
140 both data were obtained as independent tests performed within different framework. However,
141 the two tests presented provide together a well complementary view on the possible
142 methodological approaches.
143   DNA quality after extractions was evaluated using two criteria: (1) DNA yield, and (2)
144 PCR amplification success (for a detailed description see below). Here, PCR success was
145 selected as a proxy for evaluating DNA content and purity. The presence of primary and
146 secondary chemicals in plant cells are expected to have inhibiting properties on PCR reaction.
147 Hence, inadequate purification of genomic DNA, especially from polyphenols and
148 polysaccharides, could result in a lack of amplification.
149
150 *Qiagen extraction test*
151 To verify the general feasibility of obtaining PCR-amplifiable DNA from moss herbarium
152 specimens, DNeasy Plant Mini Kit was selected as a standard approach. This method was
153 selected because most of the recent bryological studies so far relied on this commercially
154 available kit (e.g. Pisa et al., 2014; Wynns & Lange, 2014; Hedenäs, 2014; 2017; Biersma et al.,
155 2017; Biersma et al., 2018a; 2018b).

156   In this test, we analysed 21 moss species of different age (12-39 years old). Isolation
157 output was tested using PCR amplification of 10 genomic loci of variable length: nuclear
158 ribosomal DNA (5.8*SR-ITS2*, *18S*, *adk* and *phy*2), and plastid marker regions (*psbAF-trnHR2*,
159 *atpI-atpH*, *trnL-trnF*, *rps4*, *atpB1-rbcL1*, *psbB-clpP*). Genetic studies using herbarium specimens
160 often highlighted the degraded nature of ancient DNA. Hence, when the above PCR tests were
161 negative, we additionally analysed selected short fragments of the plastid *trnS-trnF* region.
162   The total DNA concentration was measured in all samples tested using Invitrogen Qubit
163 2.0 Fluorometer (Life Technologies, USA) with the Qubit dsDNA High Sensitivity Assay Kit.

164 *CTAB extraction test*
165 In the second step of the tests, we compared the DNeasy Plant Mini Kit based isolation with
166 modified CTAB extraction protocols, less costly and potentially yielding a higher amount of
167 isolated DNA. Here, we used 7 different moss species which were collected over a period of 6-
168 20 years.
169   To check the quality of the extracted genomic DNA, PCR amplification was performed
170 for genetic regions of the nuclear ribosomal (*ITS5*bryo-*ITSC*bryo, *ITSD*bryo-*ITS4*bryo), and
171 plastid (*trnT-trnF*, *rps4*) DNA regions. Within CTAB extraction protocols, the type of
172 precipitation solutions, i.e. ethanol (C_2H_5OH) combined with the sodium chloride (NaCl), and
173 isopropanol (C_3H_8O), as well as the proportions of the ethanol/sodium chloride used in relation
174 to total sample volume, were the key determinants to test the effects on downstream molecular
175 applications. We proposed a modified proportion of ethanol/sodium chloride component (here,
176 protocol CTAB-ethanol/NaCl[b]), differing from the method used by Healey et al. (2014) (here,
177 protocol CTAB-ethanol/NaCl[a]). The modification applied is supposed to increase DNA purity
178 although possibly decreasing DNA concentration. Thus, we have checked whether DNA purity
179 or concentration is more relevant for obtaining PCR-amplifiable DNA from herbarium moss
180 tissue.
181   CTAB-based methods often provide a weakly purified DNA with contaminants having
182 inhibitory effects on downstream enzymatic treatments, thus we attempted to additionally purify
183 our CTAB extracted samples. To this end, we used the Genomic DNA Clean & Concentrator-10
184 kit (Zymo Research, USA) according to the manufacturer's recommendation. With this protocol,
185 we used 10 μL of input genomic DNA. Following purification, DNA was eluted from the matrix
186 with 15 μL of the DNA Elution Buffer preheated to 65°C. Usually, the Zymo-Spin matrix
187 absorbs approximately 5 μL volume of the DNA Elution Buffer and the final output was around
188 10 μL of the purified genomic DNA. In the PCR reactions, we utilized two types of genomic
189 DNA samples, before and after cleaning on the Zymo-Spin matrix, to compare PCR success rate
190 between samples with and without purification. Although silica binding based protocols provide
191 extractions of highly purified DNA samples, Qiagen DNA isolates were additionally purified
192 using Zymo-Spin matrix, in order to allow for a comparison of the final results within this assay.
193   In this test, Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) an automated
194 on-chip electrophoresis system, was used to evaluate the size distribution of the DNA fragments.
195 For this purpose, the Agilent High Sensitivity DNA kit was selected to provide the optimal

196   separation of the potentially fragmented DNA. The samples were analyzed following the
197   manufacturer's protocol.
198   DNA yield was measured using an Invitrogen Qubit 2.0 Fluorometer (Life
199   Technologies, USA) together with the Qubit dsDNA High Sensitivity Assay Kit.

PCR amplification and gel electrophoresis

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

Sequence analysis

214   Successful amplification products from Qiagen extraction test were treated using enzymatic
215   purification with ExoSAP-IT kit (Affymetrix, USA). We mixed 1 μL of template DNA with 3
216   μL of ExoSAP-IT solution, and incubated this mixture at 37°C for 15 min, and at 80°C for
217   subsequent 15 min. Cycle sequencing reactions (3 min. 96°C, 30 cycles (10 sec. 96°C, 5 sec.
218   50°C, 2 min. 60°C)) were carried out in an Mastercycler Nexus thermocyclers (Eppendorf,
219   Germany) using the BigDye Terminator 3.1 chemistry (Thermo Fisher Scientific, USA) along
220   with the BDX64 Enhancing Buffer (MCLAB, USA) in accordance with the manufacturer's
221   protocol for 32x dilution. However, in this case, several duplicate samples were taken to test
222   more fold dilutions, here 64x and 128x. To sum up, all dilutions tested resulted in high-quality
223   data with no significant differences between the obtained sequences. Cycle sequencing reactions
224   were conducted using primers used for PCR. Sequencing reactions were separated in the Applied
225   Biosystems 3130 Series Genetic Analyzer (Thermo Fisher Scientific, USA). The sequence
226   dataset was aligned with Geneious v.10.1.3 (Biomatters Ltd.), using default settings. GenBank
227   accession numbers are listed in Table S3.
228

Results

Qiagen extraction test

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

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

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

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

258   *Locus length and the PCR and sequencing success*
259   After PCR optimization, it was possible to amplify five or more of the target regions in 12 out of
260   25 studied specimens. A total of 80 sequences (product sequencing in two directions) were
261   obtained from an assay of 10 DNA loci. Four of the target regions were the most difficult to
262   amplify, with PCR success rates in the range of 16-24% for the nuclear *18S*, *adk*, *phy2* genes,
263   and 40% for the chloroplast *psbB-clpP* region. These markers are the longest target regions
264   selected for this study (all region length ca. 1000 bp). In the order of PCR success, the following
265   genetic regions can be specified: nuclear *5.8SR-ITS2* (68%; ca. 450 bp), and plastid *psbAF-*
266   *trnHR2* (68%; ca. 250 bp), *trnL-trnF* (60%; ca. 450 bp), *atpB1-rbcL1* (52%; ca. 650 bp), *rps4*
267   (48%; ca. 650 bp), and *atpI-atpH* (44%; ca. 550 bp) (see Fig. 1).
268   With regard to success of bidirectional product sequencing from obtained amplicons, it
269   amounts to: 0% for *18S* and *adk* gene, 30% for *psbB-clpP*, 67% for *phy2* gene, 87% for *trnF-*
270   *trnL*, 91% for *atpI-atpH*, 92% for *rps4* gene and *atpB1-rbcL1*, and 100% for *psbA-trnH*. The
271   quality of DNA sequences was mostly high and ranged from ca. 250 bp for *psbA-trnH* to ca.

272   1000 bp for *psbB-clpP* regions. The good quality single-strand data were also obtained in a few
273   cases, and they are marked in Table 2 with an asterisk.
274   Despite short fragments were targeted, using selected fragments of *trnS-trnF* region,
275   samples that failed into PCR reaction previously (i.e. *Andreaea depressinervis*, *A. nitida*,
276   *Sanionia uncinata*, *Schistidium sp.*) still did not amplify.

277   **CTAB extraction test**
278   The efficiency of DNA extraction using CTAB-ethanol/NaCl[a], CTAB-ethanol/NaCl[b], CTAB-
279   isopropanol, and Qiagen procedures are compared according to PCR success (Fig. 4). Four DNA
280   fragments (nuclear *ITS5*bryo-*ITSC*bryo, *ITSD*bryo-*ITS4*bryo, and plastid *trnT-trnF*, *rps4*), were
281   amplified before and after additional purification using Zymo-Spin matrix. The comparison of
282   four extraction methods showed differences in the number of successfully amplified target
283   regions. The extraction methods of CTAB-ethanol/NaCl[b] with a modified proportion of
284   ethanol/NaCl components and Qiagen had the best overall PCR success. However, when we
285   compared CTAB-ethanol/NaCl[b], and Qiagen extraction, the method that yielded the most
286   amplifiable DNA before purification was CTAB-ethanol/NaCl[b], whereas, after purification
287   treatment, the best performance showed Qiagen extraction. Remaining tested protocols, CTAB-
288   ethanol/NaCl[a], and CTAB-isopropanol had significantly worse performance, in particular before
289   additional DNA cleaning.
290   We found that additional purification and concentration using Zymo-spin matrix
291   significantly improved the PCR output in all DNA extractions methods tested. The highest PCR
292   success increase was reported in the CTAB-isopropanol method (an increase of 40,6%). For the
293   remaining DNA extractions, the success of PCR amplification after Zymo-spin matrix
294   purification was increased by 28,2% for the Qiagen, 21,9% for the CTAB-ethanol/NaCl[a], and
295   9,4% for the CTAB-ethanol/NaCl[b] procedure. Furthermore, an improvement of the genomic
296   DNA concentration was observed after using Zymo-Spin kit through all extraction methods
297   tested (Table 4).
298   The length of the target region appeared to strongly influence the amplification success.
299   Accordingly, the *trnT-trnF* locus was the longest (ca.1500 bp), and the most difficult to amplify.
300   Nevertheless, the Qiagen extraction method in combination with Genomic DNA Clean &
301   Concetrator-10 kit has proved to be the most effective for the successful amplification of the
302   abovementioned genetic region. Remaining loci (*rps4* ca. 600 bp; *ITSD*bryo-*ITS4*bryo ca. 450
303   bp; *ITS5*bryo-*ITSC*bryo ca. 380 bp) were comparable with respect to PCR success with a small
304   advantage for nuclear *ITS* regions.
305   The electropherograms obtained by automatic fragment sizing within all extraction
306   methods showed a broad distribution of bands which has indicated that genomic DNA was
307   highly fragmented (Fig. 5, 6, 7). The average sizing of DNA isolate did not vary significantly
308   across methods and is ranged from ca. 400 to 500 bp. Despite the fragment size distribution in all
309   electropherograms remains comparable, the most similar shape of the genomic DNA profiles can
310   be observed among CTAB-ethanol/NaCl[b], and Qiagen extraction method, which could be also
311   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

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

356 **Effects of target amplicon size and specimen age on successful PCR**
357 In our tests of extraction protocols, the length of the selected target regions was correlated with
358 the PCR amplification success. This appears an obvious tendency for highly degraded genomic
359 material and our results are in agreement with Särkinen et al. (2012) and Do & Drábková (2017)
360 who indicated that the most easily amplifiable DNA fragments from herbarium material were
361 those below 500 bp. In our report, based on Qiagen extraction test, the best-performing locus are
362 *psbA-trnH* (ca. 250 bp), *trnL-trnF* (ca. 450 bp), and *5.8SR-ITS2* (ca. 450 bp). The most
363 pronounced decrease in PCR success was observed in amplicons around 1000 bp (*18S*, *adk*,
364 *phy2*, *psbB-clpP*, including *trnT-trnF* region from CTAB extraction test), and was more evident
365 in nuclear regions. However, it was possible in some cases to amplify target genomic regions of
366 up to 1500 bp. Thus, even though amplification success declines with target amplicon size for
367 herbarium-based isolates, some collections may provide DNA quality high enough to provide
368 adequate data for molecular analyses. Since short fragments prevail in herbarium DNA, it is
369 expected that PCR of smaller regions has a higher success rate. It is, admittedly, under
370 abovementioned observations, but on the other hand, an attempt to amplify short, barcode
371 regions using samples which failed previously in PCR reaction (within Qiagen extraction test)
372 was still unsuccessful. In a case like this, DNA un-purity may play a more significant role in
373 inhibiting PCR reaction than DNA fragmentation. Possibly, in this particular case, PCR
374 optimisation, using both fresh and herbarium material may result in improvement of successful
375 amplification.
376 　　　　　Although in the Qiagen extraction test we did not have an equal share of specimens for
377 a given age, we found no correlation for the age of specimen and PCR success in the age range
378 examined. Successful amplification rate was comparable for the oldest (39 years old) and
379 youngest specimens (12) and rather other factors affecting the collection history seem decisive.
380 Previous studies have also shown that age of herbarium samples had no significant effect on
381 PCR success, pointing out the importance of locus types to be amplified rather than the age
382 (Särkinen et al., 2012; Do & Drábková, 2017). Summarizing, the age of moss specimen should
383 not deter bryologists from their usage in molecular research although certainly at the sample age
384 much exceeding those tested here the impact of DNA fragmentation may gradually appear
385 preponderant.

386

387 **The rate of DNA degradation in moss herbarium material – CTAB test**
388 Our extraction tests also took into consideration the level of DNA fragmentation in moss
389 herbarium samples. The overall strand breaks of DNA retrieved from the selected moss
390 herbarium specimens was high and only slightly varied between applied extraction methods, and

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

Conclusions

410 Our report is the first to offer a ready-to-use CTAB-based DNA extraction protocol tested
411 specifically for moss herbarium specimens. This procedure provides a good alternative to
412 expensive commercial kits, without negatively influencing experiment success. According to our
413 tests, the quality and quantity of DNA obtained with this method is high enough for downstream
414 PCR-based genetic analysis. Our observations regarding factors which influence the usage of
415 moss herbarium material for DNA isolation are congruent with previous studies based on other
416 groups of organisms. DNA purity and targeted amplicon size are more correlated with PCR
417 success than DNA yield. We also showed that examined genomic DNA was highly fragmented,
418 as typical for collection material, but degradation was not correlated with collection age.
419   Our observations were tested on an array of moss herbarium materials including a large
420 taxon sampling and collection age. Thus, methodological conclusions could be directly adaptable
421 to various molecular studies on mosses based on herbarium material. This seems of special value
422 when taking into account that mosses are main elements of flora in many geographical areas
423 difficult to reach due to field work logistics constraints. Antarctica and the austral polar region,
424 in general, can serve as the prominent example. In such cases, the possibility to efficiently
425 include herbarium specimens in investigation appears of key importance.

Acknowledgments

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.

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

476   Fraser CI, Connell L, Lee CK, Cary SC. 2018. Evidence of plant and animal communities at
477   exposed and subglacial (cave) geothermal sites in Antarctica. *Polar Biology* 41(3): 417-421.
478
479   Fraser CI, Terauds A, Smellie J, Convey P, Chown SL. 2014. Geothermal activity helps life
480   survive glacial cycles. *Proceedings of the National Academy of Sciences* 201321437.
481
482   Gaudeul M, Rouhan G. 2013. A plea for modern botanical collections to include DNA-friendly
483   material. *Trends in Plant Science* 18(4): 184-185.
484
485   Goff LJ, Moon DA. 1993. PCR amplification of nuclear and plastid genes from algal herbarium
486   specimens and algal spores. *Journal of Phycology* 29(3): 381-384.
487
488   Hänni C, Brousseau T, Laudet V, Stehelin D. 1995. Isopropanol precipitation removes PCR
489   inhibitors from ancient bone extracts. *Nucleic acids research* 23(5): 881.
490
491   Healey A, Furtado A, Cooper T, Henry RJ. 2014. Protocol: a simple method for extracting next-
492   generation sequencing quality genomic DNA from recalcitrant plant species. *Plant*
493   *methods* 10(1): 21.
494
495   Hedenäs L. 2014. Intraspecific genetic variation in selected mosses of Scandinavian interglacial
496   refugia suggests contrasting distribution history patterns. *Botanical journal of the Linnean*
497   *Society* 176(3): 295-310.
498
499   Hedenäs L. 2017. Three molecular markers suggest different relationships among three
500   Drepanocladus species (Bryophyta: Amblystegiaceae). *Plant Systematics and Evolution* 303(4):
501   521-529.
502
503   Hills SF, Stevens MI, Gemmill CE. 2010. Molecular support for Pleistocene persistence of the
504   continental Antarctic moss Bryum argenteum. *Antarctic Science* 22(6): 721-726.
505
506   Höpke J, Brewer G, Dodsworth S, Ortiz EM, Albach DC. 2019. DNA extraction from old
507   herbarium material of Veronica subgen. Pseudolysimachium (Plantaginaceae). *Ukrainian*
508   *Botanical Journal* 75(6): 564-575.
509
510   Hopple JrJS, Vilgalys R. 1999. Phylogenetic relationships in the mushroom genus Coprinus and
511   dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal
512   subunit RNA: divergent domains, outgroups, and monophyly. *Molecular phylogenetics and*
513   *evolution* 13(1): 1-19.
514
515   Höss M, Pääbo S. 1993. DNA extraction from Pleistocene bones by a silica-based purification
516   method. *Nucleic acids research* 21(16): 3913.
517

518 Jankowiak K, Buczkowska K, Szweykowska-Kulinska Z. 2005. Successful extraction of DNA
519 from 100-year-old herbarium specimens of the liverwort Bazzania trilobata. *Taxon* 54(2): 335-
520 336.

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

523 Kalmár T, Bachrati CZ, Marcsik A, Raskó I. 2000. A simple and efficient method for PCR
524 amplifiable DNA extraction from ancient bones. *Nucleic Acids Research* 28(12): e67-e67.
525
526 Kato K, Arikawa T, Imura S, Kanda H. 2013. Molecular identification and phylogeny of an
527 aquatic moss species in Antarctic lakes. *Polar biology* 36(11): 1557-1568.
528
529 Klavina L. 2015. Polysaccharides from lower plants: bryophytes. *Polysaccharides: Bioactivity*
530 *and Biotechnology* 145-160.
531
532 Kļaviņa L, Bikovens O, Šteinberga I, Maksimova V, Eglīte L. 2012. Characterization of
533 chemical composition of some bryophytes common in Latvia. *Environmental and Experimental*
534 *Biology* 10: 27-34.
535
536 Lavoie C. 2013. Biological collections in an ever changing world: Herbaria as tools for
537 biogeographical and environmental studies. *Perspectives in Plant Ecology, Evolution and*
538 *Systematics* 15(1): 68-76.
539
540 Lehtonen S, Christenhusz MJ. 2010. Historical herbarium specimens in plant molecular
541 systematic - an example from the fern genus Lindsaea (Lindsaeaceae). *Biologia* 65(2): 204-208.

542 Li SP, Ochyra R, Wu PC, Seppelt RD, Cai MH, Wang HY, Li CS. 2009. Drepanocladus
543 longifolius (Amblystegiaceae), an addition to the moss flora of King George Island, South
544 Shetland Islands, with a review of Antarctic benthic mosses. *Polar Biology* 32(10): 1415-1425.
545
546 Marko S, Aneta B, Dragoljub G. 2001. Bryophytes as a potential source of medicinal
547 compounds. *Pregledni članak - Review* 21(1): 17-29.
548
549 McDaniel SF, Shaw AJ. 2005. Selective sweeps and intercontinental migration in the
550 cosmopolitan moss Ceratodon purpureus (Hedw.) Brid. *Molecular Ecology* 14(4): 1121-1132.
551
552 Ochyra R, Bednarek-Ochyra H, Lewis Smith RI. 2008. New and rare moss species from the
553 Antarctic. *Nova Hedwigia* 87(3-4), 457-477.
554
555 Ochyra R, Lewis Smith RI, Bednarek-Ochyra H. 2008. *The illustrated moss flora of Antarctica*.
556 Cambridge: Cambridge University Press.
557
558 Peat HJ, Clarke A, Convey P. 2007. Diversity and biogeography of the Antarctic flora. *Journal*
559 *of Biogeography* 34(1): 132-146.
560

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

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

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

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

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

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

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

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

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

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

594 Saługa M, Ochyra R, Żarnowiec J, Ronikier M. 2018. Do Antarctic populations represent local
595 or widespread phylogenetic and ecological lineages? Complicated fate of bipolar moss concepts
596 with Drepanocladus longifolius as a case study. *Organisms Diversity & Evolution* 18(3): 263-
597 278.

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

602 Savolainen V, Cuénoud P, Spichiger R, Martinez MD, Crèvecoeur M, Manen JF. 1995. The use
603 of herbarium specimens in DNA phylogenetics: evaluation and improvement. *Plant Systematics*
604 *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 65⁰C 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 65⁰C 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 37⁰C 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 (-20⁰C) 95% ethanol and mix gently by inversion. Incubate at -20⁰C for 60 min. Note: do not leave the sample at -20⁰C 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 (-20⁰C) isopropanol to the transferred aqueous phase and mix gently by inversion. Incubate at -20⁰C 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 (-20⁰C) 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 (-20⁰C) 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*

2 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

3 Islands); POSS (Île de la Possesion, Îles Crozet); * single-stranded read or unsuccessful sequencing

4

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.

1

| 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

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

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5

6

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.
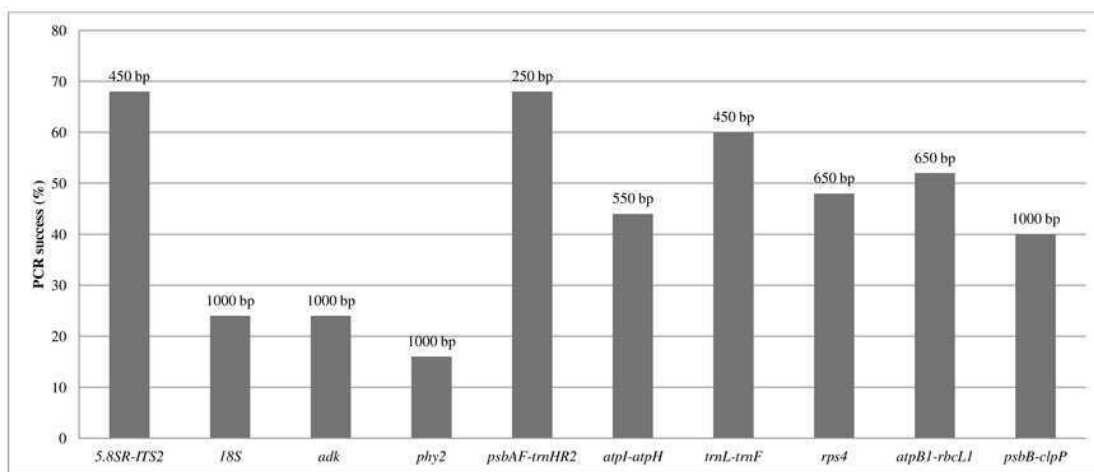
Figure 2

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.
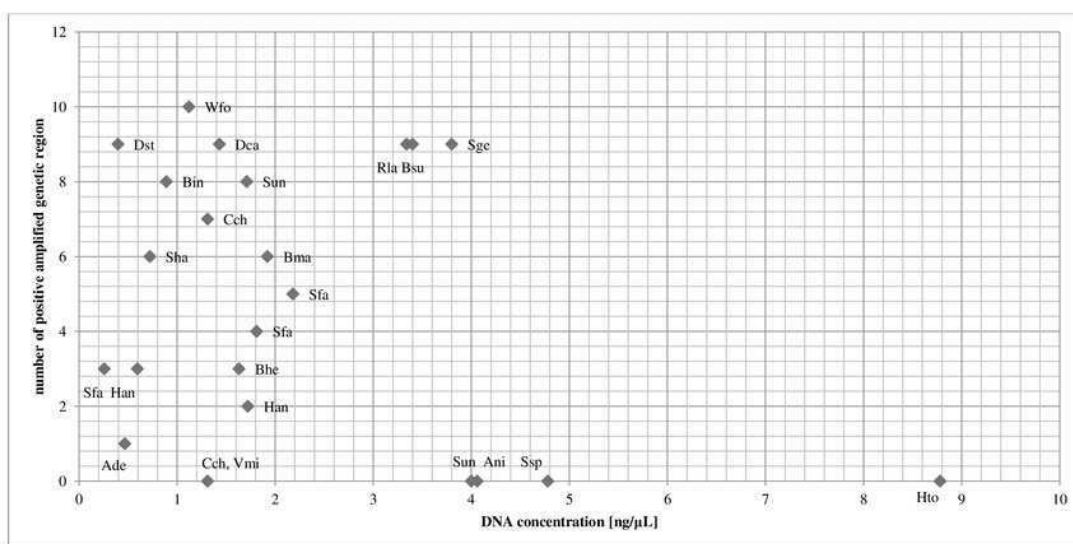
Abbreviattion used: Ade (*Andreaea depressinervis*); Ani (*Andreaea nitida*); Bhe (*Bucklandiella heterostichoides*); Bin (*Breutelia integrifolia*); Bma (*Blindia magellanica*); Bsu (*Brachythecium subplicatum*); Cch (*Cratoneuropsis chilensis*); Dca (*Distichium capillaceum*); Dst (*Ditrichum strictum*); Han (*Hymenoloma antarcticum*); Hto (*Hymenoloma tortifolium*); Rla (*Raconitrium 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).
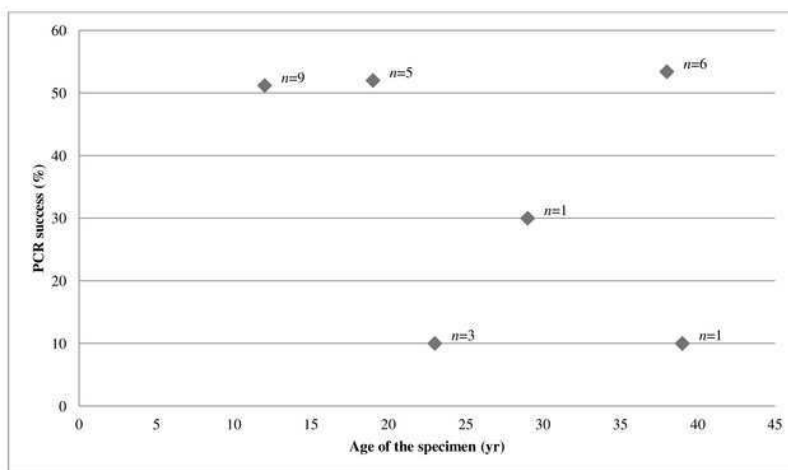
Figure 4

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.
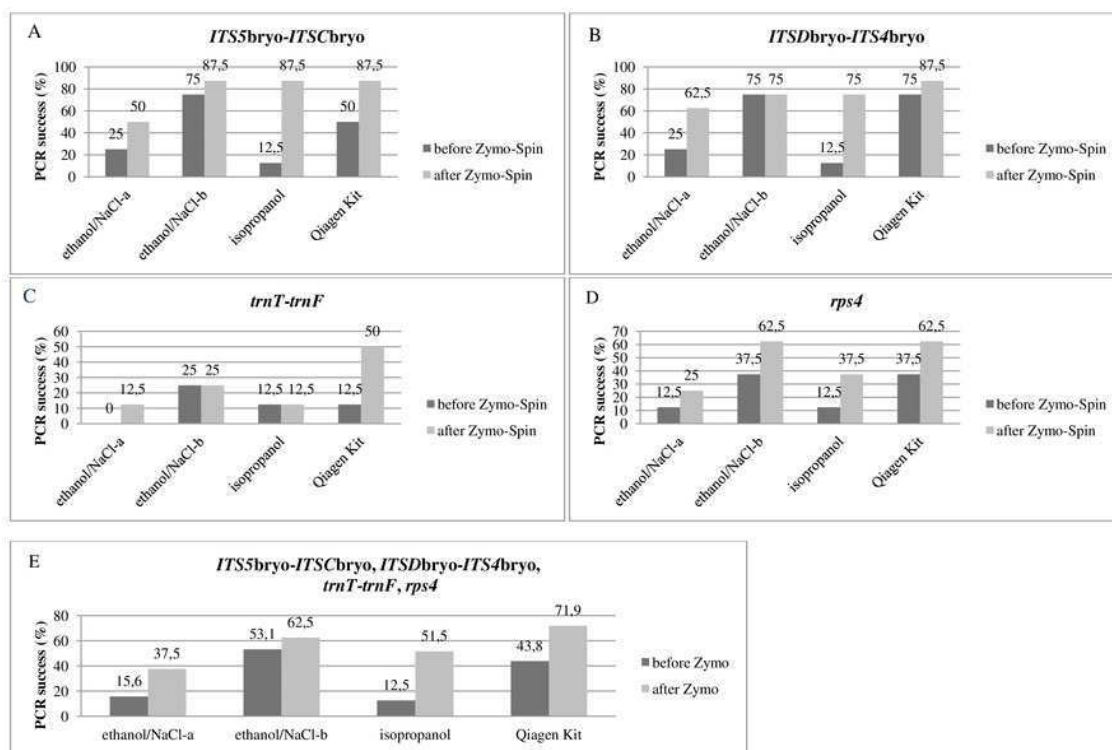
Figure 5

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.

Brachythecium rutabulum 1363/99
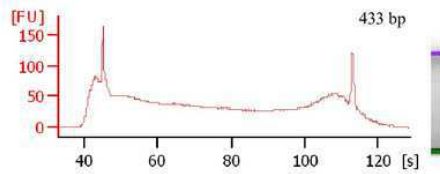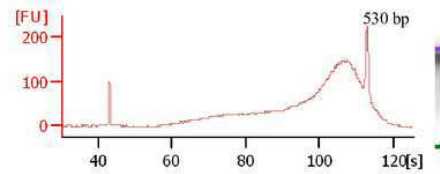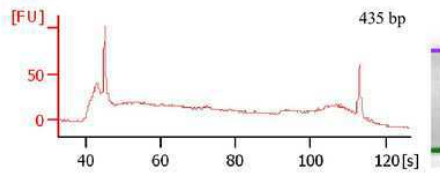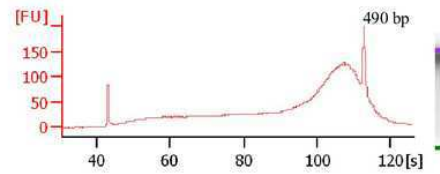
Breutelia integrifolia 3597/06
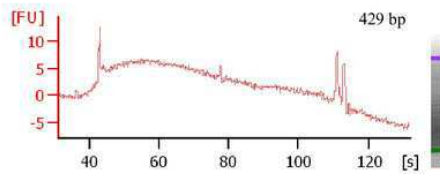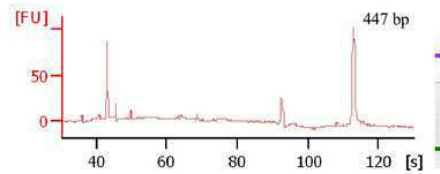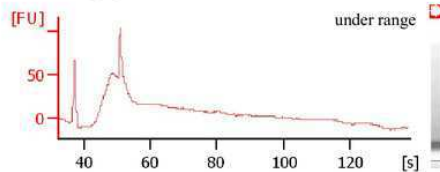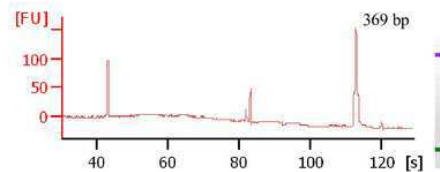
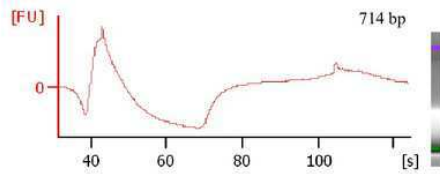*Bucklandiella stratipila* 3758/06
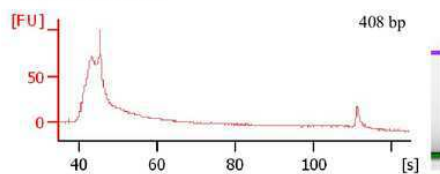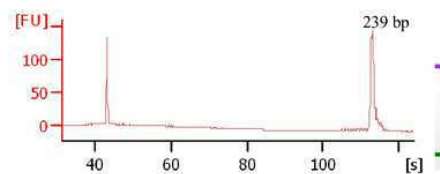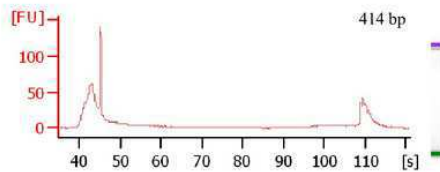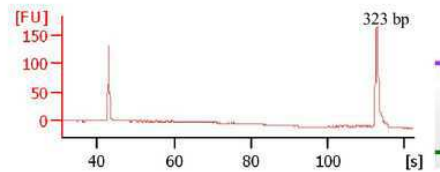
Figure 6

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

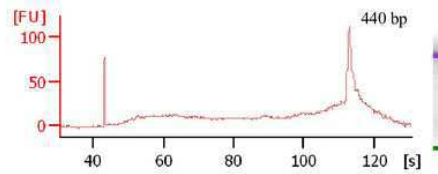**Abbreviation used: [FU]-fluorescense units (DNA amount), [s]-seconds.**

Cratoneuropsis chilensis 403/99
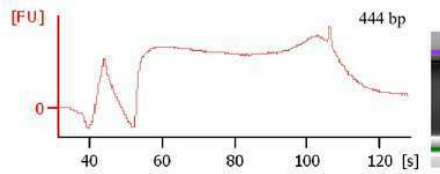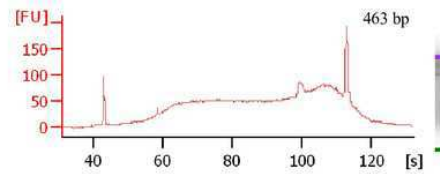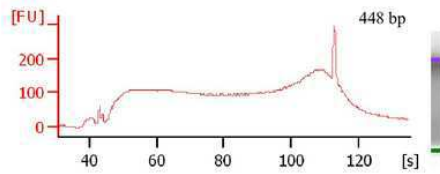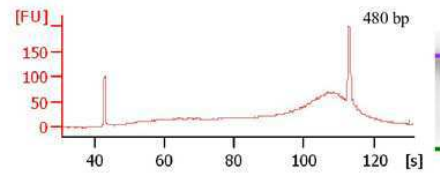
Holodontium strictum 3581/06
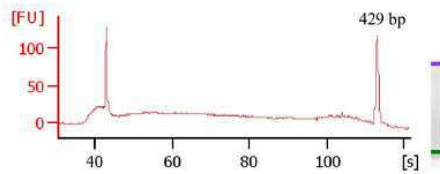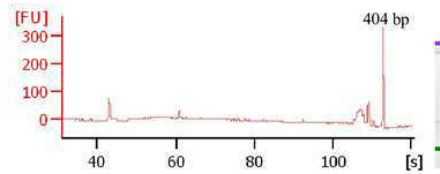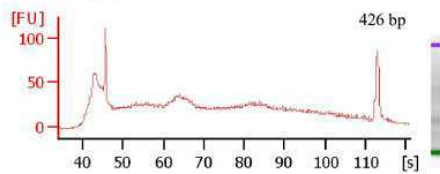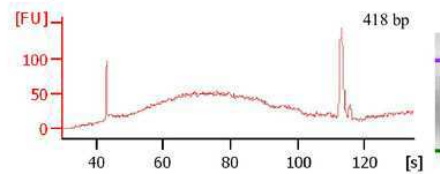
Rhacocarpus purpurascens 613/13
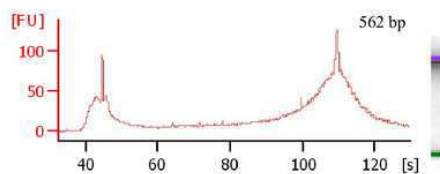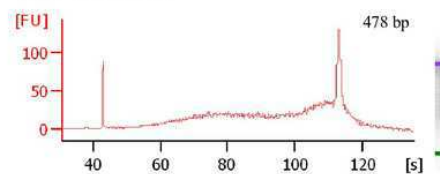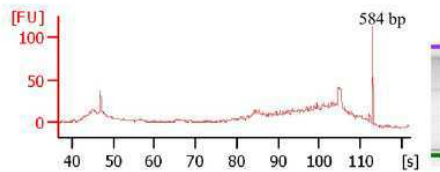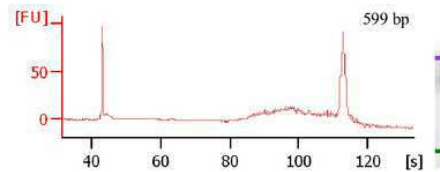
Figure 7

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.
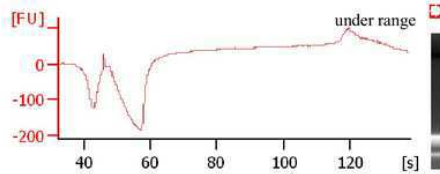
Valdonia microcarpa 555/99