Ecological Indicators 71 (2016) 388–397

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Molecular and chemical characterization of a *Sphagnum palustre* clone: Key steps towards a standardized and sustainable moss bag technique

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ARTICLE INFO

Article history:
Received 18 January 2016
Received in revised form 21 June 2016
Accepted 23 June 2016
Available online 26 July 2016

Keywords:
Atmospheric pollution
Biomonitoring
Moss bags
Trace elements
DNA molecular markers

ABSTRACT

This work aimed to define the molecular and chemical signature of a *S. palustre* clone developed in the framework of the EU-FP7 Mossclone project to improve the standardization and reliability of the moss-bag technique. The molecular characterization was performed by a set of DNA molecular markers (RAPD, ISJ, PCR-RFLP, sequencing and microsatellites) to tag the clone produced within the project. Molecular characterization also provided new DNA markers that can be applied in systematic analyses of *Sphagnum*, and gave new insights to implement well established techniques. The elemental composition of the clone was measured by ICP-MS analysis of 54 major and trace elements, with and without commonly applied pre-exposure treatments (oven devitalization and EDTA washing). Concentrations of almost all analyzed elements were significantly lower (from 10 to 100 times) in the clone than in conspecific field moss, apart from some elements (K, Mo, P and Na) deriving from the culture medium or EDTA treatment. Oven devitalization and EDTA washing did not significantly affect the clone composition. A comparison between the elemental composition of the clone with that of naturally growing *Sphagnum* species proved the particularly low elemental content of the clone. Therefore, in view of a rigorously standardized moss-bag protocol for the monitoring of persistent atmospheric pollutants, the use of the *S. palustre* clone, a biomaterial with very low and constant element composition, and homogenous morphological characteristics is strongly recommended.

1. Introduction

Air pollution monitoring and management has been one of the main European scientific and political concerns since the 1970s. Three directives were adopted by EU for air quality assesment and management (1999/30/EC, 2002/3/EC, 2004/107/EC and 2008/50/EC) relating to metals, polycyclic aromatic hydrocarbons, ozone, sulphur dioxide, nitrogen oxides and dioxide, particulate matter in ambient air. A Clean Air Policy Package (CCEP-COM/2013/0918) was adopted in December 2013, with new air quality objectives up to 2030.

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http://dx.doi.org/10.1016/j.ecolind.2016.06.044
1470-160X/

Mosses, either used as native species or as transplants (moss bags), can accumulate airborne inorganic and organic pollutants, representing a cost effective and reliable tool for air monitoring, also combined with automatic devices and emission inventories (Adamo et al., 2008a; Spagnuolo et al., 2013; Harmens et al., 2015; Iodice et al., 2016). The biomonitoring with moss bags allows to evaluate the atmospheric deposition of persistent air pollutants in a well-constrained time period, in areas lacking native species such as urban and industrial environments. It has the great advantage that can be standardized at each step, from species selection to post-exposure treatments. As a rule, the moss exposed in bags is harvested in pristine areas; however, significant differences in baseline element contents and in accumulation performance exist among different species and even in the same species grown in different habitats, or in the same area but collected in different periods (e.g. Zechmeister et al., 2003; Couto et al., 2004; Tretiach et al., 2011). The standardization of the moss bag technique is one of the most pressing and crucial concern of biomonitoring (Ares et al., 2012) and an essential prerequisite is the morphological and chemical homogeneity of the exposed material. In this sense, devitalizing and EDTA-washing treatments are recommended during moss preparation (Ares et al., 2014). Devitalization by oven drying prevents moss deterioration and enables the efficiency of contaminant capture to remain constant, as capture is mainly due to passive uptake processes that are independent of the vitality of the moss (Adamo et al., 2007; Giordano et al., 2009; Fernández et al., 2010). The use of chelating agents such as EDTA enhances the release of metals bound to cation exchange sites (Lodenius and Tulisalo, 1984). EDTA washing in moss transplants decreases element content in pre-exposure biomaterial, making it more sensitive to environmental pollution inputs (Iodice et al., 2016). In the framework of the FP7 European project Mossclone, we firstly investigated the surface properties related to metal accumulation by four devitalized moss species widely used for biomonitoring purposes (González and Pokrovsky, 2014). *Sphagnum* sp. showed the highest uptake capability and afterwards, Beike et al. (2015) selected and axenically cloned *Sphagnum palustre* L., a species allowing in photobioreactors the production of a suitable biomass for bag preparation. Recently, the *Sphagnum* clone was studied in terms of adsorption capacity of Cu and Zn (González et al., 2016), revealing its promising use as biomaterial in moss-bag technique.

This work aimed to define (i) the molecular and (ii) chemical signature of the *S. palustre* clone developed within the FP7 European project Mossclone. The molecular characterization was performed by a set of DNA molecular markers to tag the clone. The elemental composition of the clone was estimated in relation to commonly applied pre-exposure treatments, such as oven devitalization and EDTA washing, and compared with that of naturally grown *Sphagnum* species.

2. Materials and methods

2.1. Molecular characterization

Two different lines of the cloned moss *S. palustre* named 2a and 12a (Beike et al., 2015), and a reference field sample of *S. palustre* (FS) collected in Posta Fibreno (central Italy, 41°41′42.69″N, 13°41′29.98″E, 290 m a.s.l.; Terracciano et al., 2012) were analyzed. In order to compose a clone-specific molecular tag we selected and applied several techniques among those suggested for molecular markers in mosses (e.g. Crespo Pardo et al., 2014). Although the highly preserved DNA of *Sphagnum* involves some difficulties in the detection of polymorphisms at sub-specific levels, three DNA regions were selected among barcoding candidate sequences suggested for mosses (Liu et al., 2010); in addition, both unilocus and multilocus techniques were applied.

Total genomic DNA was extracted using Dneasy Plant Mini Kit (Quiagen) following the manufacturer instructions. The different procedures for each technique are described below.

2.1.1. RAPD (Random Amplified Polymorphic DNA) and ISJs (Intron-exon splice junctions)

RAPD amplifications were performed according to the protocol reported in Skotniki et al. (1999), modified for the annealing temperature (40 °C instead of 35 °C). Two 5′-FAM (blue fluorophore) labeled primers (ISJ 04 and ISJ 10, see Sawicki and Szczecińska, 2007 for further details) were selected to obtain two characteristic multiband patterns. The reactions were performed in a final volume of 20 μl, containing 40 ng of genomic DNA, 1 U Taq polymerase, 10xPCR buffer (Fermentas, USA), 200 μM of each dNTP and 20 pmol of primer. The amplification protocol provided for a hot start (1 min at 94 °C), followed by 44 cycles including the steps: denaturation at 94 °C for 1 min, annealing at 52 °C and 56 °C for 1 min for the primers ISJ 04 and ISJ 10, respectively, and elongation at 72 °C for 80 s. A further final extension at 72 °C for 5 min completed the PCR programme. Amplification products were separated by capillary electrophoresis in an ABI Prism 3730 Genetic Analyzer (Applied Biosystem); fragments were visualized as an electropherogram profile and size determinations were made by GeneMapper ver. 3.1 Software (Applied Biosystem).

2.1.2. Sequences

The chloroplast regions *matK*, *rbcL* and *trnH-psbA* were amplified. The amplification products were purified (GFX PCR DNA and Gel Band Purification Kit – Amersham Biosciences – and sequenced by BigDye Terminator ver. 3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequence reactions were run in an ABI Prism 3730 Genetic Analyzer (Applied Biosystems); electropherograms were edited and aligned in Bioedit ver. 7.1 to obtain consensus sequences. The GenBank accession numbers of the sequences are KJ865421, KJ865420 and KJ865419, respectively.

Five anonymous sequences were also developed by RAPD/ISJ reliable amplification products. Amplified bands were excised from the agarose gel and purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences); fragments were ligated into a bacterial vector using TA Cloning Kit Dual Promoter – pCR II (Life Technologies) and used to transform *Escherichia coli* DH5α. After transformation, white colonies were picked and transferred to the PCR amplification mixtures (20 μl) and to a fresh LB plate for a replica.

2.1.3. Microsatellites

Fifteen primer pairs (Shaw et al., 2008), indicated as 1, 3, 4, 5, 9, 10, 14, 17, 18, 19, 20, 22, 28, 29 and 30, were used for microsatellite amplifications. According to the different size range of the products, one of the primer for each pair was 5′-FAM or 5′-HEX labeled and five different triple reactions were prepared and amplified following the experimental procedures described in Shaw et al. (2008). Amplification products were separated by capillary electrophoresis in an ABI Prism 3730 Genetic Analyzer (Applied Biosystems); fragment profile was visualized as an electropherogram by GeneMapper ver. 3.1 Software (Applied Biosystems).

2.1.4. PCR-RFLP

The anonymous DNA region RAPDf was amplified using the F-F and F-R primers and following the protocol reported in Shaw et al. (2003). The PCR products were purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and digested by