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DNA molecular markers in mosses and their use for the characterization of a *Sphagnum palustre* clone, for its exploitation as a passive contaminant sensor

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Riassunto

Il mio progetto di dottorato s'inserisce nel progetto europeo FP7- MOSSCLONE, finalizzato allo sviluppo di un clone di muschio per il biomonitoraggio dei livelli di contaminanti nell'ambiente mediante la tecnica delle moss bags. Il mio compito è stato quello di sviluppare marcatori genomici per la caratterizzazione molecolare di questo clone.
Nel corso dei primi due anni di attività ho svolto un'accurata ricerca bibliografica sulla caratterizzazione molecolare delle piante e in particolare dei muschi. Al fine di scegliere i marcatori molecolari più adatti, ho focalizzato l'attenzione sullo sviluppo di diversi macatori, sia multiclocus (i.e. ISSR, RAPD e ISJ) che unilocus (i.e. sequenze di DNA, SCAR, SSR). Questi marcatori, dapprima saggiati su specie di muschi possibili candidati allo sviluppo del clone, quali *Hypnum cupressiforme* e *Sphagnum palustre*, sono stati successivamente saggiati su un clone di *S. palustre* prodotto nel Plant Biotech Laboratory di Friburgo diretto dal Prof. Ralf Reski. Il clone è stato caratterizzato sulla base di 15 loci SSR, di una regione anonima analizzata mediante PCR-RFLP, tre sequenze di DNA e per la produzione di banding patterns in elettroforesi capillare mediante ISJ marcati con fluorocromo. Inoltre 5 regioni anonime a partire da ISSR e ISJ sono state analizzate da bande escisse, purificate e clonate, allo scopo di sviluppare marcatori SCARs per la caratterizzazione del clone, ma anche con l'obiettivo di utilizzare queste regioni come marcatori anonimi in studi di filogenesi e di popolazione sugli sfagni.
Nel percorso del mio terzo anno ho lavorato a Freiburg, Germania, nella ottimizzazione di tecniche di coltura per il muschio acquatico *Rhynchostegium riparioides* e cercando di isolare protoplasti di *Sphagnum palustre*. In questa specie, l'isolamento di una quantità sufficiente di protoplasti, che successivamente devono rigenerare, ha lo scopo di stabilire una coltura axenica di protonemi. L' obiettivo di coltivare *Rhynchostegium riparioides* è invece quello di sviluppare una metodologia di biomonitoraggio per i sistemi acquatici continentali. Il mio lavoro è stato quello di valutare la diversa produzione di biomassa di questo muschio in funzione delle diverse condizioni di coltura.

Summary

The control of the air quality is often assessed by the use of terrestrial mosses. However, due to the lack of standardization in the used techniques, the comparison between the available studies is difficult. My PhD thesis was related to the European project FP7-Mossclone. The aim of this project is the development of a standardized method for biomonitoring the level of atmospheric pollution through the use of a devitalized moss clone of *Sphagnum palustre*, using the moss bags technique. My particular task was to perform the molecular characterization with DNA molecular markers of the clone, in order to label it for a subsequent patent development. First a bibliographical research of the application of DNA molecular markers in moss studies was performed and published. Furthermore, I have applied the acquired knowledge of the molecular techniques into a particular case: the *Hypnum cupressiforme* complex, focalizing the study in the distinction between *Hypnum lacunosum* and *Hypnum cupressiforme* using the intron-exon splice junction (ISJ) marker. I present as well some experiments that will help to optimize some cell culture techniques towards biotechnology and biomonitoring. Particularly, I studied some culture techniques in the aquatic moss *Rhynchostegium riparioides*, I studied some aspects concerning the protoplast isolation protocol in *Sphagnum palustre* and I have identified and sequenced a *Sphagnum palustre* sequence from the actin family.

General introduction and justification

Air quality and biomonitors

The interest in the environmental health, the economical concerns for exploitations and conservation require the knowledge of the status of the natural systems. In particular, the control of the levels of pollutants in the atmosphere is a key issue. Indeed air pollution has been one of main European scientific and political concerns since the 1970s.

Nowadays a Clean Air Policy Package was adopted in December 2013, consisting in a new programme for Europe with new air quality objectives for the period up to 2030. It includes a revised National Emission Ceilings Directive with stricter values for the six main pollutants (i. e., ozone, particulate matter, carbon monoxide, nitrogen oxides, sulphur dioxide and lead). On the other hand, the European Directive 2008/50/EC emphasizes the importance of the standardization of the measurement techniques and design of "sampling protocols". The final objective is then to perform accurate measurements of the air quality in order to calculate the exposure of the population and ecosystems to the contaminants. Currently the measurement of some of the contaminants cited in this directive are easily and accurately measured (mainly CO, SOx, NxOy, PAHs and particulate matter). However, other pollutants (e.g. As, Cd, Hg, Ni, Pb) present technical difficulties, and the measurements are expensive (Ares *et al.*, 2012, and references therein). Indeed the concentrations of these pollutants are often under the quantification limit.

Usually, the set of methodologies to quantify contaminants, like the control through real time measurements (i.e. hours or days) are based on physicochemical techniques. Nevertheless the use of accumulator organisms can make possible the control of this trace elements.

Biomonitoring (i.e. the control of the changes in the environment, through the use of sensitive organisms that show quantifiable responses to that changes) is one of the used approaches, and has several advantages: less funding is needed, less sampling error in intermittent point sources of contamination, study of bioaccumulation and biomagnification of pollutants in tissues and organisms and evaluation of synergistic effects in sensitive organisms, among others.

Historically, different taxa and tissues have been used to monitor the air quality. Some examples are bird feathers and organs, small vertebrates, invertebrate organisms like mollusks, different species of plants and lichens... Bryophytes in particular have several advantages to study the air quality above other taxa: i) absence of roots and cuticle (they

depend on nutrients and water present in the atmosphere); ii) high accumulation capability; and iii) well known ability to intercept inorganic and organic airborne contaminants due to high surface to mass ratio. In summary, they have been proved to accumulate a large variety of airsperse pollutants (De Nicola *et al.*, 2013; Spagnuolo *et al.*, 2013).

Traditionally, either passive or active biomonitoring techniques with mosses have been implemented. The first consist in the use of the native mosses of one or more sampling sites to assess its status, whereas in the latter transplanted mosses are used. It is important in both cases to select properly the sampling sites (e.g. taking in account the dominant winds) and to set up correctly the sampling procedure (e.g. how many samples and sub-samples for each site, distance to the punctual and diffuse sources of contamination; see as an example, Fernández *et al.*, 2007).

Although native species are still largely used for monitoring air quality in natural/rural areas, active biomonitoring by the moss bags technique claims to be a simple, reliable, and inexpensive alternative that overcomes the lack of native mosses in some sampling sites (for instance, in urban and industrial areas). Moreover a large number of different compounds can be measured at the same time, and it is a not dependent electricity technique.

Mosses can accumulate pollutants as alive or dead biomonitors without changing their performance (Adamo *et al.*, 2007), because they are able to intercept and retain particulate matter (i.e. the larger fraction of the total uptake) (Spagnuolo *et al.*, 2009b). The use of devitalized moss drastically reduce the variability in the uptake of the contaminants, because the metabolic / regulatory processes are canceled.

The moss bag technique can be standardised at each step, from species selection, to post-exposure treatments. Usually mosses exposed in bags are harvested in remote areas, far from pollution sources, and then transplanted into potentially contaminated sites. However, differences in accumulation performance and in baseline pollutant contents, may be observed depending on the species and the provenience of the moss (Ares *et al.*, 2012), which makes unfeasible to compare different monitoring studies.

However, this technique has some disadvantages (for instance, the moss has to be exposed for relatively long periods).

The Mossclone Project

The aim of the "Mossclone Project", developed within the European FP7 framework, is to produce a moss clone, which will lead to the standardization of the biomonitoring with transplanted mosses, particularly through the use of the moss bags technique. The large scale production of this biotechnological tool will provide a sustainable source of matrix to control the air quality.

Among the different objectives of the project are: i) the selection of a moss species that will be cultivated on the basis of its use as air pollution active biomonitor, and its pollutant accumulation capacity; ii) the isolation, cultivation and definition of the optimal cultivation conditions for a large scale production; iii) the molecular characterization of the obtained moss clone with the aim to register a patent; iv) the multi-element characterization of the obtained moss clone to know the initial concentrations present in the moss prior to its use as air pollution monitor; v) the physical-chemical characterization of the obtained moss to know and understand how and how much pollutants are retained by the moss clone; vi) establishment of standardized exposure protocols.

Some of the objectives are already accomplished. The species considered as suitable candidates (i.e. the most frequently used as biomonitors) were *Sphagnum palustre*, *Hypnum cupressiforme*, *Pseudoscleropodium purum*, *Brachythecium rutabulum*, *Rhynchostegium riparioides*, *Hylocomium splendens* and *Pleurozium schreberi*.

The accumulation ability of different species was already investigated (Ares et al, 2011; González and Pokrovsky 2014). Besides their growth capacity in axenic *in vitro* cultures was studied under different laboratory conditions. Finally *S. palustre* was finally selected as the most suitable species, because of its easiness of cultivation, production, and for its capability of pollutants interception.

Sphagnum species are the main components of the peatlands. This ecosystems have an enormous importance in the global climate, since they fix large amounts of carbon. Besides peatlands cover about of the 3% of the Earth land surface. Even if its distribution is worldwide, in South Europe the remaining populations are in regression, being object of special protection by the European Council Habitat Directive (92/43/EEC).

All the produced clone derive from the spores of a single sporangium capsule of *S. palustre* plant from Northern Germany. The establishment of the moss clone *in vitro* cultures and the parameters of the large scale production in photobioreactor are given in Bleike *et al.*, 2014. The production of biomass was optimized preserving the morphology and the interception ability.

Molecular characterization

The main objective of my PhD thesis was to provide an accurate molecular characterization of the produced *S. palustre* moss clone in order to register a patent. The use of biological and biotechnological tools has to be obviously associated to the accurate identification of the used material.

Broadly, the aim of the molecular characterization is to provide the specific tools / techniques in order to distinguish / identify a particular taxa. In the reviewed bibliography, the term molecular characterization is often used for the characterization of proteins or genetic regions. However, not much is written about the characterization of organisms (at least with this particular name), and the studies are mainly focused in the diagnosis techniques (e.g. the diagnosis of some strains or varieties with economic or conservation value). This diagnosis techniques are focused in the implementation of molecular techniques and markers that are able to distinguish between different taxa or even between different strains.

The basal position of Sphagnopsida (i.e. it is the older class inside Bryophyta) determines the technical approach. It is not a static class, but many interesting evolutionary processes (i.e. alopolyploidization, hybridization, change of genetic material among wide dispersed populations) indeed happens. However, the DNA of this organisms is very conserved, and therefore the use of a wide set of different techniques is mandatory.

The molecular characterization in my PhD thesis is referred to the accurate definition of the organism from the molecular point of view, using a set of different PCR molecular markers, described in the Chapter IV. In order to select the most suitable markers to carry out the molecular characterization, a review of the most commonly used DNA markers applied in mosses was assessed in the Chapter II. The Chapter III shows a brief example of the application of the intron exon junction markers to a particular case: the phylogenetic relationships among the *Hypnum cupressiforme* complex, one of the considered species in the early stages of the moss clone project. The Chapter I describes as an introduction some of the experiments carried out in my period in the University of Freiburg, always related with trials towards the optimization and implementation of some *in vitro* cultures, towards its application in both biomonitoring and biotechnology fields.

General objectives

i) The performance of some trials and the discussion of some *in vitr*o culture techniques, in order to apply them in both biotechnology and biomonitoring fields

ii) The review of the most commonly used DNA molecular markers based on PCR methods, carried out on moss studies

iii) The application of molecular markers to the case of the *Hypnum cupressiforme* complex

iv) The molecular characterization of the *Sphagnum palustre* clone through the use of DNA molecular markers

Chapter I. Preliminar trials of culture establishment in candidate species

In vitro culture optimization in Rhynchostegium riparioides

Freshwaters are often contaminated by trace elements, especially metals. The concentration of this pollutants is frequently under the detection limits. However, it is possible to use accumulator organisms, increasing the body burden and making the measurements easily assayable.

Aquatic mosses are a valuable tool to biomonitor the inorganic contamination in rivers (Gonçalves *et al.*, 1992) due to: i) its ability to accumulate high amounts of contaminants without being killed; ii) integrated record of intermittent "pulses" of pollution; iii) simplicity of sampling; and iv) easy handling and cheap chemical analysis.
There is a long tradition in the use of *Rhynchostegium riparioides* as biomonitor of the aquatic systems (e.g. Wehr *et al.*, 1983 and Wehr *et al.*, 1987; and more recently Cesa *et al.*, 2009). Some features of *Rhynchostegium riparioides* (e.g. the uptake characteristics of some elements, Claveri, 1994) have been widely studied in the laboratory. Besides, both active and passive biomonitoring have been performed with this species.

As in the moss bags technique with terrestrial mosses, there is a lack of the standardization methodologies applied in freshwaters, making impossible the comparison of the different studies. In an analog way to the "moss clone project", the establishment of an optimized axenic culture of one suitable moss species would be a first step towards the large scale production of a sustainable biomonitor for fresh water systems.

There are many factors affecting the growth rate of aquatic mosses. For instance, the survey carried out by Wehr and Whitton (1986) indicates the preference (or at least the occurrence) of the moss for/in neutral or rather basic pH values. Actually, in this survey, just a few sample sites (SS) under a pH of seven units were found / taken in account (the pH average was 7.8 units; n= 105 sites studied).

The species is usually present in waters with fast currents (Smith, 2004). So, it is evident that the current flow plus the low temperatures of England and Ireland result in much oxygenated waters. These oxygenation conditions can be reproduced in the aerated flasks. It also seems likely that *Rhynchostegium riparioides*is absent entirely from streams with low pH values or very soft water (Wehr and Whitton 1983). Furthermore, it seems that

there is a positive correlation between the content of Calcium in the ecosystem and the physiological status of the species (Wehr and Whitton 1986). As well it seems to be important the role played by the Na:Ca ratio (there is a strong negative correlation).

The main objective of this part of the PhD was to optimize the cultivation conditions for the moss *Rhynchostegium riparioides*, in order to increase the biomass production as a first step towards the development of a standardized method to biomonitoring fresh water systems.

Material and Methods

1) Preliminary experiments

The media and conditions for the preliminary experiments are shown in the Table 1.1. The aim of these experiments was to obtain a first orientation about the behavior of the cultures against different media, pH and aeration conditions. When the regular flasks were used, a volume of 200 mL was used, whereas for aerated flask the selected volume was 500 mL. The pH varied between the media, as well as the days of exposition (see Table 1.1). The initial material was not the same for all the reactions.

2) Second experiment

The used media are shown in the Table 1.2. The initial pH was 5 for all media. The volume was 20 mL in all the cases, and the experiment was running for 50 days. Two measurements of the pH were taken (at 29 days and at the end of the experiment) (see Table 1.2 and Figure A1 in the annex).

3) Third experiment (DNA buffered)

The used media are shown in the Table 1.3. The volume was 20 mL in all the cases, and the experiment was running for 50 days. Two measurements of the pH were taken (at 29 days and at the end of the experiment) (see Table 1.3).

4) Induction of branching

The objective of this experiment was to increase the number of branches developed by the moss in culture. The moss was grown first in the different media shown in Table 1.4 (volume = 20 mL) and then transfer to aerated flasks (volume = 500 mL). The number of days was different for the different media, because of the different rate of development of

the MBS (multiple branching sites, i.e. structures full of rhizoids that lead to the formation of new branches; see Figure A2 in the annex). All the aerated flasks contained Knop medium at pH 5 (Bleike *et al.*, 2014).

The increment of length was measured in the end of the experiment (after the growth in the aerated flasks), including the lateral branches in the measurements. The number of initial main stems is indicated (at the initial time); the number of MBS and the final number of branches were measured in the end of the experiment.

5) Cultivation in sieve

The moss was cultivated in a sieve for 46 days (the first week without shaking in order to let the moss get attached to the sieve), and then in a regular shaker.

Results and discussion

1) Preliminary experiments

The preliminary experiments showed that in general, the used times of exposition were not enough to see differences in more cases (see Table 1.1). For instance, the seven first reactions were designed in order to check if there were qualitative differences between the tested media. Since it was not time enough, no differences were observed. However, it was possible to see anyway how the sugar alone had a negative effect on the moss. The best media at this point, seemed to be the *Sphagnum* medium (Bleike *et al.*, 2014) (later on, rather not) because it was taking in account just the status (i.e. the robustness of the moss). The best pH seemed to be the acid one (again, later on rather not).

The aerated flasks worked out by far better than the regular flasks. The moss grew more and it was more robust. However, if the stems were directly transfered into the aerated flasks from petri dish without a previous acclimation step (i.e. at least one week in a regular flask with Knop medium) the moss died, independently of the used media (see for instance the reactions number 13, 14 and 15 in the Table 1.1).

The initial pH values were always measured, and the final pH values were sometimes measured. The obtained results are in concordance with the results of the second and third experiments. When a medium with sugar was used, the final pH was always lower than the initial one (except for the medium number 6; this could be explained by an initial harmful effect of the very low initial pH).

2) *Second experiment*

The results are shown in the Table 1.2 (see as well the Figure A3 in the annex). The best media were Knop + ME and (Knop + ME) 20%. By far, the worst media were those with sucrose and ammonium nitrate. The sugar seems to inhibit the growth of the moss.
The pH values varied according to the status of the moss. A higher pH was indicative of a better growth and robustness of the moss. However, if a correlation scatter is built, the R^2 value is not too high if pH values are considered (see Figures1.1 and 1.2); this could be explained due to the different buffering capabilities of the different media (i.e. a media with a 20% of the content of the salts will buffer less).

3) *Third experiment (DNA buffered)*

The best media was Knop + ME pH6 buffered with MES. Therefore it can be hypothesized that the optimum pH for the moss growth is around 6.

4) *Fourth experiment (development of more branches)*

The results are summed up in the Table 1.4. It is not clear which medium performed better between F5 and F6. The best results were for F5 conditions (there was a higher number of developing branches and a total bigger length, see as well Figure A4 in the annex). However, for F6 only two initial stems were considered. Therefore further research is needed in order to obtain comparable results. The F7 sample was contaminated in the transfer to the aerated flask. The higher development of branches will lead to a higher biomass production.

5) Cultivation in sieve

The two main stems grew 3.2 and 1.8 cm, which is not quite far away from the values for the flasks (for instance, with the same medium, in the experiment number 2, the main stems grew 3.1 and 2.73 cm after 50 days). However, the moss robustness was higher when aerated flasks were used.

Conclusions and outlook

The best medium was the Knop pH 6 buffered with MES. However, further research will be desirable in order to combine the increment of the development of branches (i.e. the induction of MBS) with the cultivation in a photobioreactor with Knop pH6 buffered with MES.

Besides the use of organic compounds (i.e. amino acids or other sugars different than sucrose) have been proved to enhance the growth of some mosses (see for instance arginine for *Atrichum undulatum,* in Burkholder, 1959).

Table 1.1. Media and conditions for the preliminary experiments. Type: F, regular flask; AF, aerated flask.

N	Medium	pHi	pHf	Start Material	Start	End	Type
1	Knop	4.5	-	-	22.01.2014	30.01.2014	F
2	Knop ME	4.5	-	-	22.01.2014	31.01.2014	F
3	Knop ME Suc 0,3%	4.5	-	-	23.01.2014	06.02.2014	F
4	*Sphagnum* medium	4.5	-	-	23.01.2014	03.02.2014	F
5	*Sphagnum* medium	4.2	-	-	27.01.2014	18.02.2014	F
6	*Sphagnum* medium	2.0	2.0	-	06.02.2014	21.02.2014	F
7	*Sphagnum* medium	3.0	-	-	06.02.2014	03.03.2014	F
8	*Sphagnum* medium	4.0	3.6	-	06.02.2014	21.02.2014	F
9	Tap water	4.5	-	-	14.02.2014	19.03.2014	AF
10	Knop	4.5	-	-	25.02.2014	06.03.2014	AF
11	*Sphagnum* medium	4.2	-	-	03.03.2014	19.03.2014	AF
12	Knop pH 6.9	6.9	-	Flask	19.03.2014	02.04.2014	AF
13	Tap water	4.5	-	Flask	19.03.2014	02.04.2014	AF
14	Tap water	4.5	5.9	Petri dish	27.03.2014	02.04.2014	AF
15	Knop ME	4.1	5.0	Petri dish	27.03.2014	15.04.2014	AF
16	1:4 *Sphagnum* medium	-	4.2	AF (Tap water)	02.04.2014	15.04.2014	AF
17	2:3 Sph + $CaSO_4$	-	4.5	AF (Knop 6.9)	02.04.2014	15.04.2014	AF
18	Tap water	4.5	4.9	Flask	02.04.2014	15.04.2014	AF

Table 1.2. Media, pH after 29 days, final pH and length measurements for the second experiment. Δ lenght = final length – initial length. The length of the lateral branches is included as well in the measurements. S. medium = *Sphagnum* medium; *: the media was prepared with the same amount of sucrose and NH_4NO_3, but the amount of Knop + ME content was reduced to the 20%.

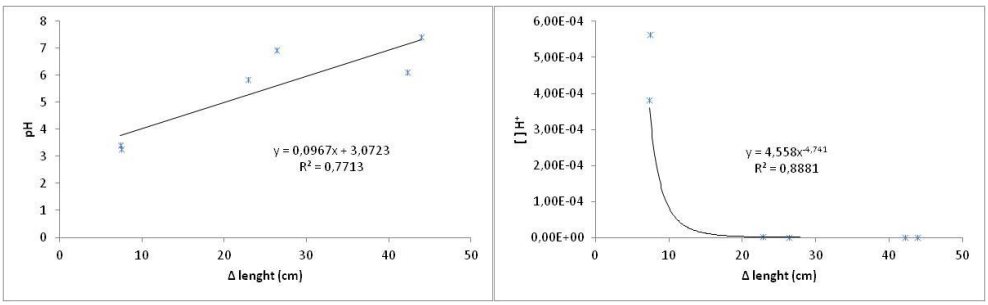
Flask	Media	Δ lenght (cm)	pH 29d	pHf
F1	Knop	22.81	5.55	5.85
F2	Knop 20%	26.37	6.51	6.92
F3	Knop + ME	42.2	5.42	6.1
F4	(Knop + ME) 20%	43.9	6.43	7.41
F5	S. medium	7.33	3.6	3.42
F6	S. medium (20%K)	7.44	3.5	3.25

Table 1.3. Media, initial pH, pH after 29 days, final pH values and length measurements for the third experiment. Δ length = final length – initial length. The length of the lateral branches is included as well in the measurements.

Flask	Media	Δ lenght (cm)	pHinitial	pH 29d	pHf
F1	**(Knop + ME) 20%**	29.35	5.00	5.57	6.7
F2	**(Knop + ME) 20% MES 1 g/L**	47.07	6.00	6.51	6.73
F3	**(Knop + ME) 20% HEPES 1 g/L**	40.54	7.00	7.19	7.11
F4	**(Knop + ME) 20%**	22.06	8.00	7.62	7.49

Table 1.4. Media, initial pH, and length measurements for the fourth experiment. Flask (LF + BF): days in Little Flask + days in Bubble Flask); Δ length = final length – initial length. The length of the lateral branches is included as well in the measurements. **NMSi : Number of initial main stems (time 0 image); MBS: Number of multiple branching sites (i.e. clear formation of rhizoid structures); NB: Number of branches. The latter parameters were observed after the growth of the moss in both the little flasks and in the bubble flasks (therefore, in the end of the experiment).

Flask	Media	NMSi + MBS + NB	Initial pH	Δ lenght (cm)
F5	Knop + sucrose (6.8 g/L)	5 + 7 + 26	5.00	22.74
F6	Knop + NH_4NO_3 (20 mM)	2 + 11 + 13	5.00	7.27
F7	Knop + sucrose (6.8 g/L)	Contaminated	8.00	-
F8	(Knop + ME) 20% + sucrose (6.8 g/L)	3 + 8 + 23	5.00	15.27



Figures 1.1 and 1.2. Scatter plot showing the increment of the length against respectively the final pH and the concentration of H^+ (mol / L) for the experiment number 2.

Protoplast isolation and regeneration in Sphagnum palustre

The protoplast isolation is an indispensable step towards the genetic modification and research in the plant systems (see Coking, 1972 and Davey *et al.*, 2005, for a review). The procedures have been optimized for a large number of different plants and tissues (see for instance Binding *et al.*, 1992). In mosses it is common to extract protoplasts from the protonema (e.g. Usui and Ito, 1985).

Since the genus *Sphagnum* circumscribes species with commercial value (for instance this genus it is currently being used in the production of some organic compounds) it would be invaluable the capability of the genetic modification of this organism in order to: i) enhance the production of some compounds; ii) optimize the excretion process; and iii) modify the chemical composition of the products.

The protonema of *Sphagnum palustre* is not available in sufficient amount in culture. Therefore, an efficient isolation protocol from the gametophytes would be desirable. Batra and co-workers (2003) successfully regenerated *Sphagnum fallax* plantlets from isolated protoplasts, using as a starting material gametophytes from a bioreactor.

There are a large number of different factors that can potentially affect to the yields of the protoplast isolation process. For instance, the concentration of the hydrolytic enzyme(s), the time of exposition and the starting material among others. The importance of the starting material consists in the different composition of the cell walls, and thus, different recalcitrance against the digestion.

The main objective of this part of the PhD was the establishment of a novel method for the efficient extraction and regeneration of protoplasts from *Sphagnum palustre* gametophytes.

Previous experiments: adjustment of the osmolarity

1) *Adjustment of the osmolarity in leaves*

The osmolarity of the enzymatic solution and regeneration medium is decisive. The scarce of cell walls make the protoplasts vulnerable against changes in the osmotic concentrations in the media. The first approach was to assess the effect of the osmolarity (always adjusted with different concentrations of mannitol) directly on the cells of the leaves of *Sphagnum palustre*. The values ranged between 400 mOsm to 1000 mOsm. In Figure A5, it is shown how the hypotonic conditions (400 mOsm) lead chlorocysts to

"occupy" the hyalocysts space, whereas at hypertonic conditions (i.e. 560 or 600 mOsm) the chlorocysts are clearly shrinking. Hence the correct osmolarity should be close to the osmolarity values in which cells are not suffering changes in volume. However, preliminary experiments have shown the futility of this approach. No protoplasts were isolated setting the osmolarity from the observed behaviours of the cells in the leaves.

2) *Adjustment of the osmolarity directly in the driselase reactions*

Different reactions with different amounts of driselase and mannitol were performed, in order to see the behavior of the protoplasts right after being released (see Figure A6 in the annex). The behaviour was not so evident because in the same samples were cells of different sizes. The osmolarities values varied between 320 and 550 mOsm. The best results were yielded for 430-435 mOsm.

Material and methods

First, a driselase solution (pectinase, cellulase and protease activity) is made. The moss is disrupted in 5 mL of mannitol (440 mOsm) until it is homogeneous (see Figure A7 in the annex). The driselase is then added until a final volume of 20 mL, calculating the desired concentration. The moss in the hydrolytic solution is set in a shaker at low speed and the digestion time is set. Afterwards the solution (with the protoplasts) is filtered two times, first with a 100 μm mesh sieve, and then, with a 50 μm mesh sieve, washing after each filtration the sieves with 5 mL of mannitol. Then a first centrifugation (500 rpm, 10 min) is made. The solution is then remove and the protoplasts (in the bottom) are resuspended in mannitol. A second centrifugation is made (500 rpm, 10 min), and finally the protoplasts are resuspended in regeneration medium. The amount of protoplast is calculated in a Neubauer chamber. The different tested conditions are given in Table 2.1.

Results and discussion of the first experiments

The number of obtained protoplasts is shown in the Table 2.1. The yield was too low, compared with *Physcomitrella patens* protonema or for *Sphagnum fallax* gametophyte numbers. Moreover, the obtained variability do not let us to distinguish or identify a key factor in the isolation reactions. There is not a correlation between the different factors and the yield of the reactions. However, no protoplasts were obtained when the moss was not disrupted. Besides higher temperatures yieded a best performance of the enzyme, but all

the obtained protoplasts were dead (see Figure A8). Thus, statistical considerations cannot be made because of the obtained variability.

Bryophytes are well known to resist extreme environmental stress. Therefore the cell walls are tough to confront extreme conditions. The protoplast isolation is easier in higher plants because once the cuticular layer is breached the cell walls of the other tissues are very easy to digest.

Even if the following parameters determine the yield of the extraction (concentration of enzyme, time of exposition and the amount of the material) it was evident that the obtained variability was not due to the conditions variation. Likely the determinant factor was the amount of cells with weaker cell walls (for instance, the amount of secondary protonema) present in cultures. Therefore, the optimization and standardization of the acclimatation and precultivation conditions prior to the performance of the protoplasts isolation reaction is required.

Changing the pre-cultivation conditions

The standardization and optimization of the cultivation conditions prior to the protoplast isolation is a key step, towards to both increasing the yield of the protoplast isolation and the protoplast rate of regeneration. Therefore the use of different precultivation media and different reaction conditions were tested. Conditions are shown in Table 2.2.

The first approach was to remove the sugar from the media and use just Knop medium, because higher contents in sugar enhance the formation of phenolic compounds (Batra *et al.*, 2003), which can be recalcitrant against the digestion by the driselase. The moss growing with low sugar concentrations was cultivated in aerated flasks to try to compensate the lack of sugar. Even with forced aeration conditions, the growth was very slow in comparaison with the optimal medium (Bleike *et al.*, 2014).

The second approach was to test the growth in previous days to the digestion reactions in dark conditions. This should potentially prevent the formation of recalcitrant compounds.

Results and discussion

The results are given in the Table 2.2. Again, the variability was too high. However, and even if the numbers were close to those of the first experiments, the amount of obtained protoplasts was double in some cases. Further research should be done. However

it does not seem very promising to focus the efforts in the direct extraction of the protoplasts from the *S. palustre* gametophytes. Therefore the next efforts should be focused in the establishment of a protonema culture, in an analog way to *Physcomitrella patens*.

Regeneration of the protoplasts

Even if the number of obtained protoplasts were low, some regeneration experiments were made with different media. The tested media were: i) Knop + ME + glucose (3.5 g/L); ii) Knop + ME + glucose (10 g/L); iii) *Sphagnum* medium without ammonium nitrate; iv) normal *Sphagnum* medium; and v) *Sphagnum* medium supplemented with double amount of sucrose. Some tests were made as well with some modified versions of the regeneration medium for *Physcomitrella patens* (knop + ME + 50 g/L of glucose + mannitol), adjusting the osmolarity at the right value for the *Sphagnum* protoplasts. The media (always set at 435 mOsm) were: i) *Physcomitrella* regeneration medium; ii) *Physcomitrella* regeneration medium with the osmolarity adjusted exclusively with glucose; and iii) *Physcomitrella* regeneration medium with 0.3% sucrose.

The five first protocols did not perform very well. In general, at 435 mOsm, the protoplasts are quite stable (i.e. in some days, they do not suffer damage or die). The main problem was that regeneration process was not triggered, and when it happened, it was too slow (see Figure A9 in the annex).

The *Physcomitrella*-based media unfortunately did not perform better. The obtained protoplasts did not resist, likely due to the protoplast isolation conditions, and no because the tested media. Therefore it is necessary further research with these media.

In the bibliography, some authors point a key issue, the decrease of the concentration of mannitol (i.e. decrease the osmolarity) to trigger the cellular divisions. The survival rate in some comparative experiments (i.e. protoplasts exposed to different media from same reactions, in order to avoid that handling or pre-cultivation conditions could affect to the final results) were neither promising. Two potential problems were found: i) the ammonium nitrate seems to be toxic / harmful in the first stages of development. This conclusion is supported by the numeric obtained results represented in the Figures 2.1 and 2.2; and ii) the amount of sugar was not enough for the first 5 tested media. Further research is needed with the *Physcomitrella* media, since the obtained protoplasts were very scarce in number.

Outlook

Buckholder (1959) described the formation of thalloid-like structures when Beijerink's media was combined with NH_4NO_3 and uracil. In the light of the obtained results seems clear that the isolation from gametophytes is a very complicated issue. Therefore the future research should be focused in the establishment of protonemal tissue, easy to digest.

Table 2.1. Protoplast isolation reactions performed in 435 mOsm. Y = the moss was disrupted prior to the digestion; N = the moss was not disrupted; M = disrupted with UltraTurrax; RT = room temperature.

Percentage driselase	Time exp (hours)	Disruption	pp / mL (Vf 10mL)	temp (°C)
0.5	16	Y	0	RT
0.75	72	Y	0	RT
1	2	N	0	RT
1	2	N	0	RT
2	2	N	0	RT
2	2	Y	40	RT
2	3	Y	0	RT
3	3	M	0	RT
3	3	Y	75	RT
3	3	Y	0	RT
3	3	Y	0	26
3	16	Y	40	26
3	5	Y	0	28
3	14	Y	0	28
3	45	Y	0	32
3	45	Y	0	35
3	3	Y	20	37
3	16	Y	100	37
4	2	N	0	RT
4	3	Y	0	RT
5	16	Y	0	RT

Table 2.2. Precultivation conditions prior to the digestion with driselase and digestion conditions. Time: days in cultivation with normal light conditions + days of total darkness; $: low light conditions; *: moss cultivated in a normal flask; §: moss washed twice with autoclaved ddwater prior to the digestion; ß: the whole cultivation process was carried out without light; **: uracil (224 mg/L) and 20 mM NH_4NO_3.

Composition	Time (days)	Percentage Driselase	Time exp (hours)	pp / mL (Vf 10 mL)
Knop pH3	15 + 3	2	4	80
$Knop pH5	17+2	3	5	110
*Knop pH5 + uracil	60	3	5	190
§Knop pH5	17+3	3	3	0
Knop pH5	12+4	3	3	180
Knop pH3**	15+3	3	3	20
Bioreactor	45	3	3h30min	0
ß *Sphagnum* medium	17	3	3h30min	20
Knop pH5**	14+3	3	3h30min	20

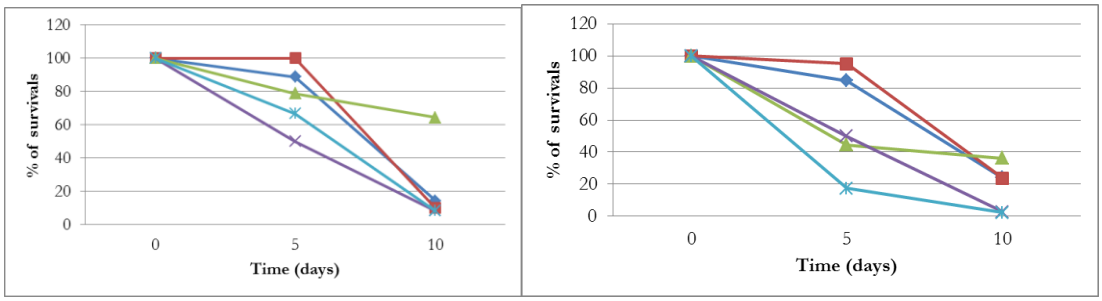


Figure 2.1 and 2.2. Regeneration experiments in *Sphagnum palustre* protoplasts from two different isolation reactions. Dark blue: Knop + ME + glucose (3.5 g/L); red: Knop + ME + glucose (10 g/L); Green: *Sphagnum* medium without NH_4NO_3; Purple: *Sphagnum* medium; Pale blue: *Sphagnum* medium with double amount of sucrose and NH_4NO_3. The osmolarity of the regeneration media was set at 440 mOsm.

Chapter II. Molecular markers in mosses

Introduction

The genome of organisms holds very useful information about taxonomy, phylogenies, biogeography, and population dynamics, with great impact in both the theoretical and practical fields of biology. Based on genetic variation, even between closely related individuals, it is possible to characterize organisms or even varieties within a species. Molecular characterization may provide a reliable and replicable tool, leading to the identification of an organism, like barcoding, and/or to the description of the evolutionary relationships among individuals/taxa. Molecular markers provide access to the enormous amount of information contained in genetic material. DNA molecular markers, or genetic markers, are identifiable DNA sequences found at specific locations of the genome, and transmitted to descendants (Semagn *et al.*, 2006). As DNA molecular markers may reveal genetic polymorphism, genome provides a new source of traits to test in systematics.

Before the introduction of molecular techniques, relationships among organisms relied on morphological traits. Phylogenetic relationships and their associated hierarchy were constructed on the basis of the evolution of homologous morphological characters, whereas in the last decades evolutionary patterns have been regarded, or even redesigned, in the light of the results obtained by DNA molecular markers. The first DNA sequence obtained for a bryophyte was the 5S gene of *Marchantia polymorpha*, as part of a study concerning the evolutionary history of plants (Hori *et al.*, 1985). The first studies specifically focused on bryophyte evolution, however, date to 1992 (Mishler *et al.*, 1992; Waters *et al.*, 1992), based on genes encoding ribosomal RNAs. The protein coding gene *rbc*L was first targeted by Lewis *et al.* (1997), while Wood *et al.* (1999) sequenced a broad array of expressed sequence tag (EST) in *Syntrichia (Tortula) ruralis*. Other techniques requiring sequence information, like microsatellites (simple sequence repeats, SSRs) and sequence characterized amplified region (SCAR) were developed for bryophytes later (Van der Velde *et al.*, 2000; Shaw *et al.*, 2003). Moreover, although any paper specifically devoted to single nucleotide polymorphisms (SNPs) in bryophyte has not been found in the present investigation, all the papers dealing with sequence data may reveal the presence of SNPs.

With reference to DNA molecular markers providing banding patterns (i.e., multilocus DNA molecular markers based on the size of anonymous loci), random amplified polymorphic DNA (RAPD) were first used in the liverwort *Porella* (Boisselier-

Dubayle & Bischler, 1994), whereas first amplifications by inter-simple sequence repeat (ISSR) primers involving bryophytes were carried out in two moss species, *Pogonatum dentatum* and *Sphagnum angermanicum*, in 2003 (Hassel & Gunnarsson, 2003), and intron-exon splice junction (ISJ) amplifications have been performed since 2005 (Polok *et al.*, 2005). Since 2000, some techniques based on the combination of PCR amplification and digestion by restriction enzymes has also been applied in bryophytes. Among these techniques, amplified fragment length polymorphism (AFLP) was used at first in a population study on *Amblystegium tenax* (Vanderpoorten & Tignon, 2000), and PCR – restriction fragment length polymorphism (PCR-RFLP), was applied in a taxonomic study within *Rhytidiadelphus* (Vanderpoorten *et al.*, 2003). Detection of RFLP using southern blotting has been used on a broad range of organisms, but this method is time consuming and requires large amounts of DNA, rarely available in small bryophytes. Additionally, RFLP may lack sufficient variation for population studies, but it has been useful in testing phylogenetic hypotheses (Boisselier-Dubayle *et al.*, 1995).

Here I provide a first review of DNA based investigations in bryophyte systematics, and assessment of the suitability of various methods for distinct research objectives. I grouped the techniques into two main categories according to the key difference of their experimental protocol, those based on actual sequence information (sequencing, SSR, SCAR), and those making inferences based on banding patterns (RAPD, ISSR, ISJ, AFLP, PCR-RFLP). The main purposes of this work are to: i) provide an overview of the molecular techniques based on PCR and utilized in mosses, specifying their advantages and drawbacks and ii) report on a large body of recent papers that illustrate their applications.

About 100 papers were examined and referenced in an extensive table focusing on the main subjects, the taxa studied, and the type of molecular marker. For an easier reading, the referenced papers were grouped into six categories, according to the main field of application: biogeography, gene expression, phylogeny, population ecology, taxonomy and technical. This table may represent a guideline for the choice of a suitable molecular technique in mosses.

Molecular techniques requiring sequence information

Sequencing

Apart from paralogous genes, sequencing gives the certainty to compare homologous loci, therefore it is more reliable than markers based on banding pattern. The

nuclear ribosomal DNA is widely used to reconstruct phylogenetic relationships (Samigullin *et al.*, 1998; Olsson *et al.*, 2009; Merget & Wolf, 2010), within families (Shaw & Allen, 2000), genera (Grundmann *et al.*, 2006; Shaw *et al.*, 2010; Carter, 2012) or species (Skotnicki *et al.*, 2004; Vanderpoorten *et al.*, 2004; Grundmann *et al.*, 2006; Draper *et al.*, 2007; Korpelainen *et al.*, 2008b; Draper *et al.*, 2011). Particularly, ITS sequences were used as markers in many studies in mosses (e.g. Korpelainen *et al.*, 2008b; Spagnuolo *et al.*, 2008; Carter, 2012; Terracciano *et al.*, 2012b). The chloroplast (cp) *trn*T-F region and especially the *trn*LUAA intron are the most widely targeted loci, not only in bryophytes, but also in other plants (Quandt & Stech, 2005). The locus has been complemented by the *rps*4 gene in many studies aiming at resolving relationships within classes and families (Buck *et al.*, 2000 and 2005; Goffinet *et al.*, 2001; Shaw & Allen, 2000; Pedersen *et al.*, 2007; Bell & Hyvönen, 2010), genera (Shaw *et al.*, 2010; Carter, 2012) and even among populations (Vanderpoorten *et al.*, 2004; Werner & Guerra, 2004; Draper *et al.*, 2011). The *atp*B-*rbc*L intergenic spacer has also been frequently used in moss phylogeography (e.g., Grundmann *et al.*, 2006; Pedersen *et al.*, 2007). The idea that the mitochondrial DNA offers few loci routinely targeted for moss phylogenetic studies is primarily due to the presumably lower variability compared with the cpDNA (Stech & Quandt, 2010). However, the *nad*5 gene, recently sequenced for many moss species (Liu *et al.*, 2012), has been used for reconstructing bryophyte phylogenies since 1999 (Beckert *et al.*, 1999), and so have other mitochondrial DNA regions (Cox *et al.*, 2010; Wahrmund *et al.*, 2009 and 2010). Nonetheless, the use of single copy genes, like all chloroplast and mitochondrial genes, in moss systematics, is strongly affected by the available knowledge about the flanking regions of the gene itself to develop suitable primers (Wall, 2002).

SSR (simple sequence repeats)

The microsatellites (also known as simple sequence repeats, SSRs) are tandem repeats of short DNA sequence motifs. These markers have several advantages: i) each locus is well-defined and codominant; ii) they are frequently polymorphic at population level, due to differences in the number of repeats; and iii) they are easily tested by PCR.

However, sequence information is required in order to design species-specific primers into the flanking regions of the repeat motif. The polymorphism of a locus is based on the number of repeats, that increases or decreases the length of the locus, and it is commonly evidenced by comparing PCR-fragment length by capillary electrophoresis. SSRs are highly abundant in eukaryotic genomes (Korpelainen *et al.*, 2007). Furthermore,

the levels of heterozygosity can be exceptionally high, even in species in which the level of genetic variation detected by other methods may be low. In this laborious technique, the markers act as a single-locus trait, and their development is expensive (Korpelainen *et al.*, 2007). These disadvantages are compensated when the protocol optimized to amplify SSRs for a given species can be extended to closely related taxa, as in *Sphagnum* (Provan & Wilson, 2006). Microsatellites have been used to evaluate the genetic diversity (e.g., Liu *et al.*, 2010a), to test species delimitation (Shaw *et al.*, 2009), to circumscribe geographic distribution of intraspecific genets (Szövényi *et al.*, 2008), to identify parental taxa in alloploids (Ricca & Shaw, 2010), or to evaluate degrees of inbreeding and its consequences (Szövényi *et al.*, 2009b; Van der Velde *et al.*, 2001 a and b). Given the versatility of these markers their use in mosses is expected to be increased.

SCAR (sequence characterized amplified region)

The SCAR markers are locus-specific markers, which are detected through specific primers designed from identified sequences obtained by other methods. For example, Shen *et al.* (2011) developed the SCAR primers from specific RAPD bands; Albani *et al.* (2004) used instead ISSR bands. Once the sequence of the locus is known, a pair of specific 15-30 bp primers is designed, making this technique suitable for diagnostic purposes, like selecting a commercially valuable crop or variety (e.g. Yang *et al.*, 2013). These markers were used in bryophytes to delimitate *Calypogeia* species (Buczkowska & Dabert, 2011) and to target three anonymous DNA regions in *Sphagnum* (Shaw *et al.*, 2003). SCAR technique provides a useful tool in order to develop new sequence markers starting from any technique based on banding pattern. However, several cloning-sequencing cycles may be needed before obtaining a suitable anonymous region to be characterized.

Molecular techniques producing banding patterns

RAPD (random amplified polymorphic DNA)

This technique is a random targeting PCR approach based on short (10 bp) primers. A possible mutation across one or more annealing sites along DNA template will result in the absence of the relative band, and subsequently in a different banding profile. This technique allows fast, easy and cheap comparison between a big set of samples when prior sequence information is not available (Kjolner *et al.*, 2004). Due to these advantages, this technique seemed suitable for populations study (Selkirk *et al.*, 1997; Skotnicki *et al.*,

2004). However, the typically low stringency conditions may lead to a low reproducibility of bands (Kjolner *et al.*, 2004). Other limitations of RAPD technique are the dominant behaviour, the lack of knowledge on amplification products, and the co-migration of fragments that are not homologous but of similar size. This technique thus calls for particular caution in applying the method and interpreting its results. Furthermore, the targeted DNA must be highly purified, because, contaminating (endophytic or epiphytic) fungi may be amplified and lead to artefactual results (Stevens *et al.*, 2007). This technique has been applied to investigate genetic variability and structure among populations, in several moss species (*Hennediellaheimii*, *Sarconeurum glaciale* and *Ceratodon purpureus*), and in particular Antarctic mosses (Selkirk *et al.*, 1997; Skotnicki *et al.*, 1998; Skotnicki *et al.*, 1999; Dale *et al.*, 1999; Skotnicki *et al.*, 2004). It was also used to study the species delimitation in *Sphagnum recurvum* (Sastad *et al.*, 1999). However, recent application of RAPD technique in moss populations, compared to SSR, showed that the first lead to an overestimation of the gene diversity due to sample contamination (Clarke *et al.*, 2009). For this reason, the RAPD technique is now used to study somatic mutation in *in vitro* cultured axenic plants (Enan, 2006).

ISSR (inter-simple sequence repeat)

ISSR technique is nearly identical to RAPD technique except that ISSR primers are designed from microsatellite regions and are longer than RAPD primers. The first ISSR studies were published in 1994, focused on cultivated species (see Wolfe, 2005 for a review). These markers are based on the amplification of DNA segments present between two identical microsatellite repeat regions, usually a dinucleotide or a trinucleotide motif, and oriented in opposite directions. Therefore, the technique uses microsatellites as primers in a single PCR reaction targeting multiple genomic loci to amplify ISSRs of different sizes. The primers can be either unanchored or, more frequently, anchored at 3' or 5' end with 1 to 4 degenerate bases extended into the flanking sequences (Weising *et al.*, 2005). ISSRs have high reproducibility, due to the use of longer primers (16-25 bp), which allow a higher stringency and more reliable and reproducible bands than RAPD (Vanderpoorten *et al.*, 2003). Several studies targeted ISSR in mosses, to estimate genetic diversity and clonal structure in populations, in relation to bottleneck/genetic drift processes (Vanderpoorten *et al.*, 2003; Gunnarsson *et al.*, 2005; Spagnuolo *et al.*, 2007a and 2009a), to habitat disturbance and fragmentation (Spagnuolo *et al.*, 2007b; Spagnuolo *et al.*, 2009b; Patiño *et al.*, 2010), and for conservation purposes (Terracciano *et al.*, 2012a). In some cases this technique was

applied to resolve taxonomic problems (Hassel & Gunnarsson, 2003; Spagnuolo *et al.*, 2008), or distinguish sexes (Korpelainen, 2008a). Furthermore, ISSR markers, similarly to RAPD, can be the basis for the development of other markers, i.e. microsatellites (Korpelainen *et al.*, 2007; Provan & Wilson, 2006), or SCARs (Buczkowska & Dabert, 2011).

ISJ (intron-exon splice junction)

ISJs are semi-specific dominant markers being present in the majority of plant genes (Gang *et al.*, 2011). The longer primers used in this technique allow a higher annealing temperature, obtaining more reproducible bands. ISJ markers were used in plants (e.g. in cotton, Gang *et al.*, 2011) but very few references were found in mosses. Sawicki and Szczecinska (2007) optimized the technique in mosses, evaluating its suitability for *Sphagnum* spp. and *Orthotrichum speciosum*, whereas Sawicki *et al.* (2012) evaluated the distinction between the subgenera *Orthotrichum* and *Pulchella* of *Orthotrichum*. Even if some sequence information was required for their initial development (Weining & Langridge 1991), all primers can be designed based on the constant oligonucleotide sequence located at the junction between intron and exon, with the addition of a short random sequence. In theory, by using a given primer pair, a researcher might also decide whether to amplify exonic or intronic regions, but in practice each primer can also act as a forward and a reverse, so the technique is comparable to the RAPD or the ISSR.

AFLP (amplified fragment length polymorphism)

This DNA fingerprinting technique was developed by Zabeau and Vos (1993) (but see also Vos *et al.*, 1995 and Vos & Kuiper, 1997) and is based on PCR amplification of selected restriction fragments of a total digested genomic DNA. AFLP markers reveal a "restriction site" polymorphism, usually made of small DNA fragments of few base pairs (up to 500) that must be amplified by PCR to be visualized. The originality of the AFLP method was to design and synthesize arbitrary primers first, and then to legate them to target DNA fragments. The AFLP arbitrary primers are called "adapters" and consist of a known sequence of about 20 nucleotides. The target DNA sequences are DNA fragments generated by restriction enzymes. Then, adapters are ligated at each end of a restriction fragment by enzyme ligase. Finally, adapters are used in a PCR as annealing sites to amplify the restriction fragments. Amplified products are separated by electrophoresis on

acrylamide gel or by capillary electrophoresis. AFLP provide reliable dominant markers, which are highly reproducible due to the specificity of restriction enzymes. The great advantage of using AFLPs in population studies is that the number of loci scored and polymorphisms identified in each reaction is high (Blears *et al.*, 1998). Moreover, these markers give an estimate of variation across the entire genome, which in turn gives a good general picture of the level of genetic variation of the investigated organism. In mosses, AFLP technique has been used to perform studies having different aims. For example, linkage maps at species level were developed (McDaniel *et al.*, 2007 and Kamisugi *et al.*, 2008), but always combined with other techniques (like SSRs) to ensure the accuracy of the results. Fernández *et al.* (2006) inferred the presence of cryptic species in *Grimmia laevigata* and Pfeiffer (2006) described the molecular diversity and clonal structure in *Rhytidium rugosum* based on AFLP data.

PCR-RFLP (PCR-Restriction Fragment Length Polymorphism)

The technique consists of a digestion by a restriction endonuclease of a specific amplified DNA segment; the presence or the absence of a given restriction site in the sequence will provide polymorphism between individuals. The technique requires specific primers for locus amplification and may be applied both to known sequences and to anonymous DNA regions; in the latter case, SCAR primers must be designed in order to obtain the amplification product. PCR-RFLP can be performed by single or double digestions; this choice is generally related to the expected polymorphism of a specific DNA segment. If expected divergence is low, then the double or triple digestions may yield more characters. PCR-RFLP is a fast and reliable technique if applied to known sequences (e.g. ribosomal genes and spacers) for which universal primers are available; it proved useful in the resolution of taxonomic problems (Vanderpoorten *et al.*, 2003). It could also provide a first information level in taxonomic studies, before sequencing, but it gives too little information, compared to sequencing, to be used in phylogeny or in biogeography; moreover, the evolution of these traits cannot be modelled properly. Due to their theoretical high level of polymorphism, RFLP of anonymous DNA regions may also be applied in population studies, but the development of specific primers may be expensive and time consuming.

Conclusions

The table enclosed in this overview may provide a guideline in the choice of suitable molecular markers to address specific questions in moss systematics. Molecular phylogenetic inferences have revealed extremely powerful for testing traditional taxonomic hypotheses, and this is especially true in taxa with rather simple morphologies like bryophytes (Vanderpoorten & Shaw, 2010), particularly when reduction in morphological complexity may obscure the phylogenetic affinities among taxa (e.g. Goffinet *et al.*, 2011).

Of course, sequences and related markers are very reliable because they compare homologous DNA loci. By contrast, a certain caution is needed while analysing molecular results from markers grounded on banding patterns; assuming that bands having the same bp length are homologous loci is the rationale of these techniques, but it is not necessarily true. In addition, the co-migration of different amplification products having the same length is a further source of artefacts. However, the critical evaluation of a large body of literature highlights that sequence-related markers are not applicable to all research fields. Although they result useful in phylogenies and population studies over wide geographic ranges, in general they may not reveal polymorphisms at small geographic scales and at low taxonomic ranks (i.e., between and within species). This makes it necessary to fall back on molecular techniques based on banding pattern. Among these, the ones requiring capillary electrophoresis, like AFLP, should be preferred because of the high reproducibility and the precision of the detection system. But also ISSR and ISJ may yield robust results if an accurate choice of reproducible bands is made, and high resolution systems for band evaluation are applied. The use of labeled primers, for example, allows for an accurate fragment separation by capillary electrophoresis. Differences in principles, methodologies, and applications of various types of molecular markers require careful consideration in choosing one or more of such methods, according to the kind of study to be undertaken.

Since a literature examination is always a good starting point, in this paper I provide an extensive compilation of molecular studies, specifically devoted to mosses, in the form of a table in which the papers are grouped under different main subjects, specifying the techniques and the taxa studied. This could be a useful tool to choose the most suitable technique and methodology according to the specific research purposes.

Biogeography

Key words	Molecular Technique	Taxa	Reference
ITS, *trnL-F*, phylogeographic relationships, glacial refugia	cp/nr DNA	*Neckera* and *Exsertotheca*	Draper *et al.*, 2011
Bioindication, floristic province	AFLP	*Grimmia laevigata*	Fernández *et al.*, 2006
Bottleneck, clonal plant, gene flow, invasive species, recombination	ISSR	*Pogonatum*	Hassel *et al.*, 2005
Allopolyploidy, interploidal hybridization, phenotypic variation	SSR	*Sphagnum cuspidatum*	Karlin *et al.*, 2011b
ITS, neighbor-joining analysis	nrDNA	*Hilpertia velenovskyi*	Sabovljevic *et al.*, 2006
Cryptic speciation, DNA sequence variation, disjunctions, liverworts	Isozymes, DNAseq	Bryophyta	Shaw 2001
Antarctica, colonization, genetic diversity	RAPD	*Sarconeurum glaciale*	Skotnicki *et al.*, 1999
Clonal diversity and structure, geographic pattern, vegetative growth	cpDNA, ISSR	*Pleurochaete squarrosa*	Spagnuolo *et al.*, 2009a
Allopolyploid, bayesian analysis, hybridization, *trnG*	cpDNA, SSR	*Sphagnum troendelagicum*	Stenoien *et al.*, 2011
Dispersal, glacial refugia, phylodemography, cp non-coding regions	cpDNA	*Sphagnum fimbriatum, S. squarrosum*	Szövényi *et al.*, 2006
GapC, ITS, sequence variability, multilocus dataset	nrDNA	*Sphagnum fimbriatum, S. squarrosum*	Szövényi *et al.*, 2007
Shared polymorphism, intercontinental migration, isolation with migration	SSR	*Sphagnum*	Szövényi *et al.*, 2008
Gametophytic selection, molecular adaptation, GDPH, GapC, Rpb2	DNAseq	*Sphagnum fimbriatum*	Szövényi *et al.*, 2009a
Genetic structure, inverse isolation hypothesis, island colonization, long-distance dispersal	SSR	*Sphagnum*	Szövényi *et al.*, 2012
Phylogeography, coalescent theory, gene flow, molecular evolution	SSR	*Sphagnum angermanicum*	Stenoien *et al.*, 2010
Gene sequences, rps4, evolution, molecular systematics	cpDNA	*Tortula muralis*	Werner & Guerra, 2004

Gene expression

Evolution, gene expression, molecular modeling, nonsymbiotic hemoglobins	DNAseq	*Ceratodon purpureus*	Garrocho-V.& Arredondo-P., 2008
ABA, codon usage, stress	EST	*Physcomitrella patens*	Machuka *et al.*, 1999

Plastid DNA, transcript profiling	Microarray	*Physcomitrella patens*	Nakamura *et al.*, 2005
Chloroplast genome sequence, gene content, polymorphism, *Physcomitrella patens*	cpDNA	*Tortula ruralis*	Oliver *et al.*, 2010
Molecular evolution, *trnT-F, trnL*, groupI intron, inversion, hairpins	cpDNA	Bryophyta	Quandt & Stech, 2004
Molecular evolution, *trnLUAA* intron, secondary structure	cpDNA	Bryophyta	Quandt & Stech, 2005
Gene knockout, homologous recombination, molecular farming	EST	*Physcomitrella patens*	Reski & Frank, 2005
Salt tolerance, transcription factors, NCED, abscisic acid	Microarray	*Physcomitrella patens*	Richardt *et al.*, 2010
Moss, MADS-box gene, MICK*-type, evolution	RT-PCR, DNAseq	*Physcomitrella patens*	Riese *et al.*, 2005

Phylogeny

Bayes factors, incongruent gene trees, phytogeography, reticulate evolution, 18S	cp/nr DNA	Polytrichopsida	Bell & Hyvönen, 2010
Morphological concepts, *trnL-F*, *rps4*, sequences	cpDNA	Pleurocarpuos mosses	Buck *et al.*, 2000
Systematics, *Timmia*, *atpB-rbcL*, *trnL-trnF*, 26S	cp/nr DNA	Timmiaceae	Budke & Goffinet, 2006
ITS, *rps4*, *psbA2*, *trnG*, monophyly, speciation	cp/nr DNA	*Scleropodium*	Carter, 2012
Hybridization, ITS, *trnG*, molecular and morphological incongruence	cp/nr DNA	*Isothecium*	Draper *et al.*, 2007
Evolution, systematics, *rbcL*	cpDNA	Orthotrichaceae	Goffinet *et al.*, 1998
Phylogenetic relationships, cryptic species, transatlantic distributions, ITS	cp/nr DNA	*Pleurochaete*	Grundmann *et al.*, 2006
Genetic diversity and structure, postglacial, dispersal history, rare species	ISSR	*Sphagnum angermanicum*	Gunnarsson *et al.*, 2005
Chloroplast sequences, systematics, *trnL-F*, *trnS-rps4*	cpDNA	Grimmiaceae and Ptychomitriaceae	Hernández-M. *et al.*, 2007
Inversions, *trnL*, Group I intron, microstructural changes	cpDNA	Grimmiaceae and Ptychomitriaceae	Hernández-M. *et al.*, 2008
Linnean shortfall, Macaronesia, morphology	cp/nr DNA, SSR	*Rhynchostegium riparioides, R. alopecuroides,* and *Gradsteinia torrenticola*	Hutsemékers *et al.*, 2012
rbcL, *rps4*, *trnL-F*, *nad5*, 18S rDNA, parsimony analyses	cp/mit/nr DNA	Polytrichales	Hyvönen *et al.*, 2004
Allopolyploidy, allotriploid gametophytes, speciation	SSR	*Sphagnum*	Karlin *et al.*, 2009

Founder effect, intragametophytic selfing	SSR	*Sphagnum*	Karlin *et al.*, 2011a
Data partitioning, monophyly test, conflict visualization, sequence	mitDNA	Funariaceae	Liu *et al.*, 2012
Linkage map, adk and phy2 genes, interpopulation cross	AFLP	*Ceratodon purpureus*	McDaniel *et al.*, 2007
Genealogical conflict, hybrid speciation, reproductive isolation, adk, apr, papr	cp/nrDNA	*Physcomitrium*	McDaniel *et al.*, 2009
Cytoplasmic inheritance, maternal inheritance	cp/mitDNA	*Sphagnum*	Natcheva & Cronberg, 2007
Evolution, ancestral character states, maximum parsimony, bayesian inference	cpDNA	Bryaceae	Pedersen *et al.*, 2007
Cytotypes, peat moss, polyploidy, allopolyploidy, homoploid hybridization	cpDNA, SSR	*Sphagnum subsecundum* complex	Ricca and Shaw, 2010
Sphagnum subsecundum complex, allopolyploidy	SSR	*Sphagnum lescurii*	Ricca *et al.*, 2011
ITS, sequence	cp/nrDNA	Bryophyta	Samigullin *et al.*, 1998
ITS, molecular taxonomy, *trnH-psbA*, genetic relationships	cp/nrDNA, ISJ, ISSR	*Orthotrichum*	Sawiki *et al.*, 2012
Morphological incongruence, geographic speciation, *trnL-trnF* , ITS	cp/nrDNA	Fontinalaceae	Shaw & Allen, 2000
Bayesian inference, bryophytes, peatmoss, phylogenetic reconstruction	DNAseq	Sphagnopsida	Shaw *et al.*, 2003
Species delimitation, recombination, gene flow, monophyly	cp/nr DNA	*Sphagnum* section *Acutifolia*	Shaw *et al.*, 2005
Long-distance dispersal, northern hemisphere biogeography, anonymous loci	ITS, LFY1/2, *trnL/G*	*Sphagnum subsecundum* complex	Shaw *et al.*, 2008a
Ascertainment bias, DNA fingerprinting	SSR	*Sphagnum*	Shaw *et al.*, 2008b
Allopolyploidy, PCR recombination, species delimitation	SSR	*Sphagnum subsecundum*	Shaw *et al.*, 2008c
Peat mosses, species delimitation	SSR	*Sphagnum atlanticum, S. bergianum*	Shaw *et al.*, 2009
Bryophyte evolution, Miocene, peatlands	cp/mit/nr DNA	*Sphagnum*	Shaw *et al.*, 2010
ITS, 26S, *psbT*, *trnL*, evolution, monophyly, phylogenetics	cp/nr DNA	Sphagnopsida	Shaw, 2000
ITS 1 phylogeny, ribosomal DNA	ITS sequences	Pottiaceae	Spagnuolo *et al.*, 1999
DNA barcoding, mitochondrial DNA, nuclear DNA, plastid DNA, phylogenetics	Review	Bryophyta	Stech & Quandt, 2010

Haplolepideous mosses, non-coding plastid markers, *trnL-F*, *rps4*	cpDNA	Dicranidae	Stech *et al.*, 2012
Gene trees, adenosine kinase gene, gene duplication, paralogy, polyploids	cp/nr DNA	*Hypnum*	Terracciano *et al.*, 2012b
Species concept, paraphyly, budding speciation, monophyly	Review	Bryophyta	Vanderpoorten & Shaw, 2010
Sequence, *trnL–trnF,atpB–rbcL*,18S–5.8S–26S, adk gen	cp/nr DNA	*Hygroamblystegium*	Vanderpoorten *et al.*, 2004
Group I and II intron, indels, intergenic region, RNA editing	cp/mit DNA	Bryophyta	Wahrmund *et al.*, 2009
Asymmetric recombination, hybridization, introgression	ISSR	*Sphagnum capillifolium, Sphagnum quinquefarium*	Natcheva & Cronberg, 2007
Group I intron cobi420, inversions.	mitDNA	Bryophyta	Wahrmund *et al.*, 2010

Population ecology

Genetic diversity, Antarctica	RAPD	*Hennediella heimii*	Dale *et al.*, 1999
ISSR protocol, population genetics, PCR techniques	ISSR	*Pogonatum* and *Sphagnum*	Hassel & Gunnarsson, 2003
Genetic structure, aquatic moss	SSR	*Platyhypnidium riparioides*	Hutsemékers *et al.*, 2008
Clonality, morphological shift, population structure, species delimitation	SSR	*Sphagnum cribosum*	Johnson *et al.*, 2012
Allopolyploid, clone, genetic diversity, long-distance dispersal, vegetative propagation	SSR	*Sphagnum cuspidatum*	Karlin *et al.*, 2011c
Clonal growth, genetic isolation, genetic richness, forest disturbance	SSR	*Acanthorrhynchium papillatum*	Leonardia *et al.*, 2012
Epiphytic bryophyte, exotic tree plantation, forest disturbance, habitat suitability	ISSR	*Isothecium myosuroides*	Patiño *et al.*, 2010
Clonal reproduction, dispersal, habitat, vegetative reproduction	AFLP	*Rhytidium rugosum*	Pfeiffer *et al.*, 2006
Genetic variation, Antarctic populations	RAPD	*Sarconeurum glaciale*	Selkirk *et al.*, 1997
Genetic variation, RAPD profiling of genetic diversity, Antarctica	RAPD	*Ceratodon purpureus*	Skotnicki *et al.*, 1998
Clonal moss, genetic diversity, *trnL* intron	cpDNA, ISSR	*Pleurochaete squarrosa*	Spagnuolo *et al.*, 2007a
Epiphytic moss, genetic structure, genetic impoverishment, habitat disturbance	ISSR	*Leptodon smithii*	Spagnuolo *et al.*, 2007b
Inbreeding, mating system, reproductive skew, selective embryo abortion	SSR	*Sphagnum lescurii*	Szövényi *et al.*, 2009b

Conservation genetics, fragmentation, habitat disturbance	ISSR	*Sphagnum palustre*	Terracciano *et al.*, 2012a
AFLP, *Amblistegium*, polymorphism, genetic differenziation	AFLP	*Amblystegium tenax*	Vanderpoorten & Tignon, 2000
Clonal structure, genotypic diversity, male fertilization success, paternity analysis	SSR	*Polytrichum formosum*	Van der Velde *et al.*, 2001

Taxonomy

Genomic relationship, molecular and morphological congruence	RAPD	*Thuidium tamariscinum, Hyophyla comosa*	Alam *et al.*, 2012
ITS, *molecular systematics, Tortula ruralis* Complex	nrDNA	*Tortula densa*	Frahm & Sabovljevic, 2006
New Zealand, Australia, alloploid	SSR	*Sphagnum section Sphagnum*	Karlin *et al.*, 2008
Allopolyploidy, eastern North America, peat mosses, species concept	SSR	*Sphagnum*	Karlin *et al.*, 2010
Allopolyploidy, long-distance dispersal, Sphagnum, taxonomy	SSR	*Sphagnum falcatulum* species complex	Karlin *et al.*, 2013
ISSR, ITS, PCR-RFLP, species concept, species delimitation	nrDNA, SSR	*Rhytidiadelphus*	Korpelainen *et al.*, 2008b
Ex situ conservation, threatened bryophytes, barcoding, *trnL-F* intron	AFLP, cpDNA	*Orthodontium gracile*	Rowntree *et al.*, 2010
Sphagnum balticum, S. lindbergii, population analysis	Isozymes, RAPD	*Sphagnum recurvum* complex	Sastad *et al.*, 1999
Genetic similarity, katG, ITS, species identification	nrDNA, ISJ, ISSR, RAPD	*Sphagnum* section *Acutifolia*	Sawiki & Szczecinska, 2011
DNA sequence analysis, ITS, within- and among-colony variation	nrDNA, RAPD	*Ceratodon purpureus*	Skotnicki *et al.*, 2004
Corsica, endemism, species concept, incongruence, morphology	cp/nr DNA	*Leptodon corsicus, Neckera besseri, Homalia webbiana*	Sotiaux *et al.*, 2009
Genetic variation, ITS, *trnL*, species concept	cp/nr DNA, ISSR	*Hypnum cupressiforme* complex	Spagnuolo *et al.*, 2008
Species-level systematics, sibling species	nrDNA, ISSR, RFLP	*Rhytidiadelphus*	Vanderpoorten *et al.*, 2003
ITS sequence data, Canary Islands	nrDNA	*Platyhypnidium torrenticola, P. riparioides*	Werner *et al.*, 2007
Haplolepidous mosses, *rps4*, *trnl-F*	cpDNA	*Hymenoloma mulahaceni*	Werner *et al.*, 2013

Technical

DNA barcoding, *rbcL*, *rpoC1*, *rps4*, *trnH-psbA*, *trnL-trnF*, sequence	cp/mit/nr DNA	Bryophyta	Liu *et al.*, 2010b

Chromosomal inversion, *Physcomitrella patens*, sex-linked loci, phylogeography	EST	*Ceratodon purpureus*	McDaniel *et al.*, 2013
AFLP reproducibility, genotyping error rate	AFLP	*Campylopus introflexus*	Mikulášková *et al.*, 2012
Peat bogs, ISSR cloning, conservation	ISSR, SSR	*Sphagnum capillifolium*	Provan & Wilson, 2007
AFLP, ISSR, RAPD, bryophytes	ISJ	*Sphagnum* and *Orthotrichum*	Sawiki & Szczecinska, 2007
Gene targeting efficiency	Genetic transform	*Physcomitrella patens*	Schaefer, 2001
Bioinformatics, EST database, plant genome	SSR	*Physcomitrella patens*	von Stackelberg *et al.*, 2006
Linkage map, genome sequence	AFLP, SSR	*Physcomitrella patens*	Kamisugi *et al.*, 2008
Marker identification, genome screening, cloning, SSR development	ISSR, SSR	Bryophyta, Algae	Korpelainen *et al.*, 2007
Gender ratio determination, sex ratio determination, issr sequence	ISSR	*Pseudocalliergon*	Korpelainen *et al.*, 2008a
Antarctic moss, gene ontology, stress response	EST	*Aulacomnium turgidum*	Liu *et al.*, 2010a
Development of microsatellite markers, population genetics	SSR	*Ptychomitrium gardneri*	Liu *et al.*, 2010b

Chapter III. A practical case: the relationship between *Hypnum cupressiforme* and *H. lacunosum*, as revealed by the intron-exon splice junction markers

Introduction

The relationships among the *Hypnum cupressiforme* complex (HCC) have not been totally solved yet. *Hypnum lacunosum* (HL) belongs to this complex, and it has been treated like distinct species or like a variety of *Hypnum cupressiforme* (HC) (see Terracciano *et al.*, 2012 and references therein). Hedenäs (2012) reported the mixed occurrence of both HC and HL in the same patch, evidencing that both morphotypes are genetically distinct.

Due to the presence of phenotypic plasticity in both members, the information given by the molecular markers is essential. Terracciano and co-workers (2012) studied the phylogenetic relationships within the HCC by sequencing intergenic spacers ITS1 and ITS2 of the nrDNA and chloroplast *trn*L_{UAA} intron; the authors found that HL cannot be distinguished from HC, based on that sequence analysis. Therefore, since the sequencing-based methods have failed so far in distinguish between both entities, it is necessary to investigate techniques with a greater resolution power (i. e. multilocus techniques).

The PCR based markers have proved their suitability to assess taxonomic relationships in bryophytes (see Crespo *et al.,* 2014, for a review); however, the selection of the more convenient marker(s) is critical.

The intron-exon splice junction (ISJ) multilocus technique (Weining & Langridge, 1991) is particularly suitable for several reasons: i) the higher annealing temperature in comparison with other PCR techniques, like RAPD, as well as the semi-specific sequence of the primers, complementary to the exon-intron junction, contribute to increase the stringency level reached in the reactions; ii) the technique is easily assayable, simple in the procedures and reproducible; iii) the sequence of the primers is not based on repetitive motifs, excluding their annealing to repeated DNA template (Sawicki & Szczecińska, 2007); and iv) ISJs were successfully used in bryophytes (Sawicki & Szczecińska, 2007, Sawicki *et al.*, 2012).

In this chapter, I use the intron-exon splice junction markers to study the relationship between HC and HL taxa.

Material and methods

Sampling

Four taxa within the HCC were included in the present study: HC, HC var. *resupinatum*, HC var. *filiforme* (HF) and HL (proveniences are given in Table 3.1). The number of specimens analysed was 11. As an additional caution to distinguish between HL and HC, the lengths of 10 stem leaves for each used gametophyte were measured and the morphological shoot type was assigned (see Hedenäs, 2012 and Zubel, 2007). Only the shoots with leaf lengths out of the overlapping range between both species were analysed. Moreover, all shoots exhibiting intermediate morphological characters were excluded.

DNA extraction and PCR amplification

Total genomic DNA was extracted by a modified cetyltrimethyl ammonium bromide (CTAB) method (Murray & Thompson, 1980). Two primers (ISJ 4 and 10) and four primer pairs (ISJ 1-4, 2-7, 3-10 and 4-9) (Sawicki & Szczecińska, 2007) were used to amplify the DNA, selected after an initial screening of 30 different combinations from a set of 12 primers. The PCR conditions are given in Sawicki & Szczecińska, 2007.

Data analysis

ISJ bands were scored manually, and 29 unambiguous and reproducible bands (all polymorphic) were selected. The length of the bands varied between 300 and 1200 bp. Electrophoretic patterns were translated into a binary data matrix and then analysed by UPGMA cluster analysis in Syntax 2000 (Podani, 2001), in order to obtain a dendrogram based on the dissimilarity between the different specimens. AMOVA was calculated by using a pairwise distance method in GenAlEx v. 6.5 (Peakall & Smouse, 2012).

Results and discussion

The relationships between the samples are summarized in the Fig. 3.1. There are two different clusters, a first only including HL and a second cluster including all the other samples , with HF forming a distinct subcluster within this heterogeneous group.

The total variance was partitioned for 28% among species, and for 72% within species.

This is the first study demonstrating a molecular differentiation between HL and HC; the previous molecular investigations focused on the HCC highlighted indeed, the distinction of *H. jutlandicum* within the complex, as well as the segregation of *H. imponens* from the complex itself (Spagnuolo *et al.,* 2008, Terracciano *et al.*, 2012). However, no informative molecular result was observed for HL so far.

The existence of two clearly defined clusters is consistent with the inference by Hedenäs (2012), who suggested the presence of two distinct entities due to some different morphological traits and to their sympatric occurrence. Indeed, our results confirm that both taxa can coexist preserving their genetic identity.

Liu and co-workers (2010) analysed 10 different barcoding sequence regions in order to test their suitability for moss species circumscription. They found that in recent evolving groups, like Hypnales, sequence divergence is not coherent with morphospecies delimitation, and high within-species variation is frequently observed. These findings are in agreement with a partitioning within species of the total variance for a good 72%; despite this high intra-specific variation, a genetic distinction between HL and HC is here observed.

Although the results clearly distinguish between HC and HL, the information given is not enough. Thus, it is necessary to increase the number of studied specimens for each species and their geographic proveniences in order to enhancethe significance of the results. In conclusion, the ISJ markers could provide a valuable tool in order to distinguish between these two taxa.

Table 3.1. Provenience of the different species.

ID	Species	Provenience
L1	*Hypnum lacunosum*	Spain
L2		Czech Republic
Hc1	*Hypnum cupressiforme*	USA
Hc2		Norway
Hc3		South Africa
Hc4		USA
Hc5		England
Hc6		England
F1	*H. cupressiforme* var. *filiforme*	USA
F2		USA
Hr	*H. cupressiforme* var. *resupinatum*	England

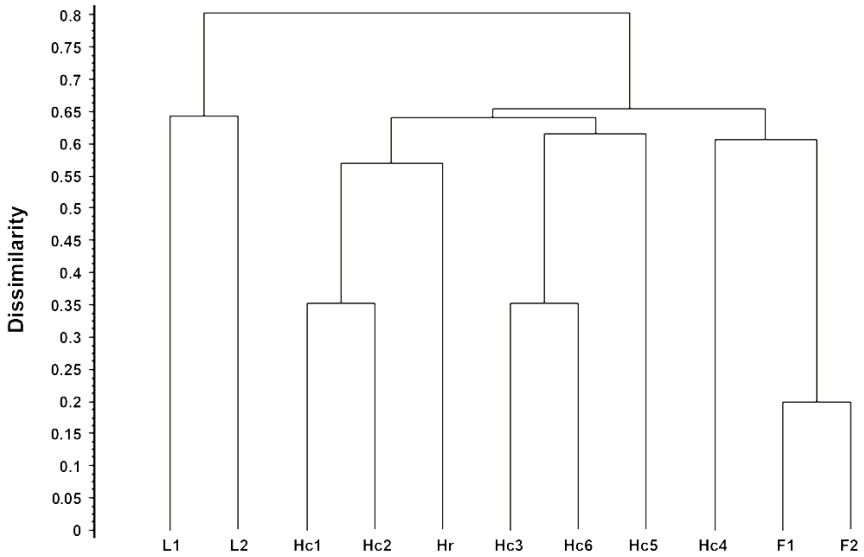


Figure 3.1. Dissimilarity tree obtained by a pairwise distance method. L: *Hypnum lacunosum*; Hc: *Hypnum cupressiforme*; Hr: *Hypnum cupressiforme* var. *resupinatum*; F: *Hypnum cupressiforme* var. *filiforme*.

Chapter IV. Molecular characterization of a peat moss (*Sphagnum palustre*) clone established in axenic culture

Introduction

Terrestrial mosses have been used to assess heavy metal contamination since the end of the 1960s (Rühling& Tyler 1968; Tyler & Rühling 1970). The particular features of these organisms (i.e. absence of roots and cuticle, high accumulation capability, and its ability to intercept inorganic and organic airborne contaminants) make them suitable biomonitors of the air quality.

Both native and transplanted mosses can be used. In the first, the differences in methodologies (e.g. chosen species, starting material and sampling strategy among others) returns non comparable results. The latter technique overcomes the scarce of moss in urban areas, but sometimes the extraction of high amounts of natural material is not possible or sustainable. The "Mossclone project" aims to resolve these problems, establishing a large scale production of a *Sphagnum palustre* clone towards a standardized and sustainable moss bag technique.

The aim of the molecular characterization (one of the objectives inside the Mossclone project) is to provide the specific tools / techniques in order to distinguish / identify a particular taxa (in this case, the clone of *Sphagnum palustre*). The use of biological and biotechnological tools has to be associated to its accurate identification.

The main objective of this Chapter was to carry out a molecular characterization of the clone of *Sphagnum palustre* selected for the moss bag preparation. The molecular traits found in the peat moss clone were compared to those found in field shoots.

The selection of the most suitable techniques was determined by following two different approaches: i) the study of multiple loci, in order to analyze at the same time different random regions of the DNA; and ii) the study of known regions, usually exhibiting a lower variability. All the techniques are reviewed in Chapter II.

Material and methods

Samples and DNA extraction

Two different lines of the *S. palustre* clone, previously established in axenic culture and produced in photobioreactors (Beike *et al.*, 2014), named 2a and 12a, were analysed. A reference field sample collected in Posta Fibreno, Italy (Terracciano *et al.* 2012) was used as well. Total genomic DNA was extracted using Dneasy Plant Mini Kit (Quiagen) following the manufacturer's instructions. The different procedures for each technique are afterwards described.

Random Amplified Polymorphic DNA (RAPD)

A total set of 28 primers was tested: OPA (1, 3, 8, 9, 13, 17), OPB (7, 8, 15), OPC (1, 4, 5, 13, 14), OPJ 19, OPP (1, 7, 16), S (62, 63, 88, 90, 343, 500, 511, 1002, 1015, 2059 (Operon Technologies). Reaction mix tubes of 25 μL were prepared (see Table 4.1). The PCR amplification protocol was performed as follows: one cycle of 3 min at 94ºC, 2 min at 40ºC and 3 min at 72ºC; 43 cycles of 10 s at 94ºC, 10 s at 40ºC and 50 s at 72ºC; and one final extension cycle of 3 min at 72ºC. Amplificates were run out in a 1.5% agarose gel.

Intron-exon splice junctions (ISJ)

Two 5'-FAM (blue fluorophore) labelled primers (ISJ 04 and ISJ 10, see Sawicki and Szczecinska 2007 for further details) were selected to obtain two characteristic multiband patterns. The reactions were performed in a final volume of 20 μL (see Table 4.1). The amplification protocol provided for a hot start (1 min at 94ºC), followed by 44 cycles including the steps: denaturation at 94 ºC for 1 min, annealing at 52 ºC and 56 ºC for the primers ISJ 04 and ISJ 10 respectively for 1 min, and elongation at 72 ºC for 80 sec; a further final extension at 72 ºC for 5 min completed the PCR program.

Amplification products were separed by capillary electrophoresis in an ABI Prism 3700 Genetic Analyzer (Applied Biosystem); fragment profile was visualised as an electropherogram by Gene Mapper Software (Applied Biosystem).

Twenty-five different combinations of both individual and pairs of non-labelled primers from a total set of 12 primers were tested as well, with the same previously explained PCR conditions. The objective was to obtain clear bands in order to purify and sequence them to design the basis primers for the development of the SCAR technique.

Sequencing / Barcoding

The chloroplast regions *trnH-psbA*, *rbcL* and *matK*, three among barcoding-candidate sequences, were amplified. The amplification products were purified (Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequence reactions were run in an ABI Prism 3700 Genetic Analyzer (Applied Biosystem); electropherograms were edited and aligned in Bioedit ver. 7.2.5 to obtain consensus sequences. The GenBank accesion numbers of the sequences are respectively KJ865419, KJ865420 and KJ865421.

Microsatellites

Primer sequences and microsatellite characteristics for the 15 markers analyzed in this study are described by Shaw *et al.* (2008a). The 15 microsatellite markers, numbered as in Shaw *et al.* (2008), are: 1, 3, 4, 5, 7, 9, 10, 12, 14, 16, 17, 18, 19, 20, 22, 28, 29, 30.
Microsatellites were amplified in 8 ml multiplexed reactions, each targeting a set of three loci. Primer sets were arrayed for multiplexing according to expected fragment sizes (for non overlapping amplification products) and alternating fluorophores. Each primer pair included a forward primer fluorescently labeled with HEX or 6-FAM (Integrated DNA Technologies, Coralville, IA). Multiplexing was accomplished using a Qiagen Multiplex PCR kit (Valencia, CA), scaled for smaller reactions, but otherwise used according to the manufacturer's recommendations. Five to 20 ng of genomic DNA in 3 ml dH20 served as template in each reaction. A standard thermocycling regime was implemented for all primer sets, with no additional optimization. This consisted of an initial denaturation and hot-start activation at 95ºC for 15 min, then 30 cycles of 94ºC for 30 sec, 54ºC for 90 sec and 72ºC for 60 seconds. A final extension at 60ºC for 30 min was performed. PCR products were diluted in sterile water, and 1.2 ml of the dilution was mixed with GS500 size standard and Hi-Di TM Formamide (Applied Biosystems, Foster City, CA) for electrophoresis on an ABI 3730 sequencer. Size determinations and genotype assignments were made using GeneMarker 1.30 software (Softgenetics, State College, PA).

PCR Restriction Fragment Length Polymorphism (PCR-RFLP)

The tested chloroplast regions were ITS, psbA-trnH and matK. Moreover, three anonymous sequences, amplified as in Shaw *et al.* (2003) were tested (RAPDa, b and f).

The anonymous DNA region RAPDf was amplified following the protocol reported in Shaw *et al.*, 2003. The PCR products were purified (Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and digested by a set of 17 restriction enzymes (Fermentas, Thermo Fisher Scientific) according to manufacturer's instructions.

Sequence Characterized Amplified Region (SCAR)

Five different anonymous regions from ISSR and ISJ bands were developed. After the purification (Illustra GFX PCR DNA and Gel Band Purification Kit, GE Healthcare), amplificates were cloned with CloneJET PCR Cloning Kit (Thermo Scientific), according to manufacturer's instructions, and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). A BLAST analysis against GenBank database revealed no close hits.

Longer primers were designed (Table 4.2) with primer3 software (bioinfo.ut.ee/primer3-0.4.0/, Untergrasser *et al.*, 2012). A standard PCR program was set as following: an initial cycle of 3 min at 94ºC, then 30 cycles of 94ºC for 30 s, 52ºC for 1 min and 72ºC for 45 seconds. A final extension at 72ºC for 7 min was performed as well. Amplificates were run out in a 1% agarose gel.

Results and discussion of the techniques

Random Amplified Polymorphic DNA (RAPD)

The height of the obtained bands is showed in Table 4.3. The amplifications were carried out in at least four different DNA obtained from different extractions. All the characteristic band patterns were clear and reproducible.

Some researchers have reported two main problems with the RAPD methodology: the low stringency conditions and the amplification of strange DNA (for instance, from "hitch-hiking" fungi, Stevens *et al.*, 2007). However, both problems were overcomed: i) the contamination with other organisms and the artifacts from the field particles are avoid by cultivating the moss in axenic conditions; and ii) the annealing temperature was 5ºC higher than in the rewieved literature (for instance Skotnicki *et al.*, 1998) (see as well Figure A10 in the annex).

Intron-exon splice junctions (ISJ)

The height of the obtained bands with labelled primers is showed in Table 4.4. Only two bands out of 9 were common to both clone lines and field samples. Therefore, seven bands can be used to characterize the *Sphagnum palustre* clone and distinguish it from field samples.

The justification of the use of this method is explained in Chapter III. The use of labelled primers leads to a better measurement of the size of the bands. And even more important, it is possible to discard co-migration events.

Microsatellite

The length of the obtained bands is showed in Table 4.5. The analysis revealed that only four loci out of 15 (corresponding to primers 5, 9, 14, 17) are different between the clone and the field sample. Two primers (4 and 18) did not yield products.

Microsatellites have been previously used to perform phylogeography analysis in peat mosses. For instance, Shaw *et al.* (2008) developed 30 microsatellite primers that have been widely used afterwards in a high number of different species (see for instance, Ricca & Shaw, 2010 and Shaw *et al.*, 2009). Due to the low level of molecular variation, the microsatellite primer pairs developed for a single species were able to amplify several different taxa through the genus.

PCR-RFLP

The double digestion of the RAPDf region with *Hinf*I - *Hind*III and the single digestions with *Eco*RI and *Mnl*I derived in characteristic, reproducible band patterns for the clone (see Figures A11 and A12 in the annex). The other restriction endonucleases provided no polymorphisms.

PCR-RFLP is a specially suitable technique to reveal polymorphisms between individuals (Crespo *et al.*, 2014). Even if the information given by this techniques is lower than sequencing, the possibility of the development of diagnosis protocols surely compensate this drawback. It is noteworthy that the same technique applied to several nuclear and plastid DNA regions usually considered for sequence analyses, or even to other two anonymous regions, called RAPDa and RAPDb, did not provide any polymorphisms within *S. palustre*.

Sequence Characterized Amplified Region (SCAR)

The five designed primers (Table 4.2) amplified the moss clone. Even if the single amplifications do not characterize the moss clone (actually the primers yielded optimal amplifications in other species, like *S. magellanicum* and *S. contortum* among others), the sequences will potentially uncover polymorphisms. This novel markers (no positive hits were found in a BLAST analysis against the GenBank database) could be potentially used in both population or species-specific level. Thus further research is needed in order to understand the degree of variability of these novel sequences.

Conclusions

The clone of *Sphagnum palustre* was succesfully characterized with six main DNA molecular techniques. A total number of 24 loci were studied and characterized. Besides 23 bands from multiloci techniques have been proved to be reliable markers for the molecular characterization purpose.

Table 4.1. Quantities in the PCR reaction tubes for ISSR, RAPD and ISJ techniques.

Technique	ddH2O	Buffer (10x)	dNTPs (25 mM)	Primer (50 µM)	Taq (1U)	DNA (20 ng/µL)	Final volume
ISSR	13,8	3	2	1	0,2	1	25
RAPD	19,5	2,5	1,25	1	0,2	1	25
ISJ	13	2	2	0,4	0,2	2	20

Table 4.2. Sequences of the SCAR designed primers. Tm: Melting temperature; E size: expected size of the PCR amplification product.

Primer	Sequence (5' - 3')	Tm	E size
S40_fw1	TTTTCCACATACACCACCGC	58.8	350
S40_rv1	AGTTAACGTTACCCAGGCGA	59.0	
S42_fw1	ACGTCGGCTCTCAGGTATTC	59.3	400
S42_rv1	CTTCGTTGTGGGGTCTGTTG	59.1	
S44_fw1	GCAGTAATTGATCTTGGCAACC	58.2	250
S44_rv1	TGCACTGCCAAAAGTTTCAG	57.4	
T31_fw1	ACCACCACCACGCATAGAG	59.4	425
T31_rv1	AAATGTGTTGAAGACCCCATGA	58.2	
T37_fw1	CGCATTCACAGGGCTCTAAC	59.0	580
T37_rv1	AGCTTGTAACGAAGGGACCT	58.7	

Table 4.3. Characteristic band profiles for RAPD primers OPB15, OPJ19 and S88. Table 4.4. Characteristic band profiles for ISJ-FAM labelled markers ISJ4 and ISJ10. FS: field sample.

Primer bands (bp)		
OPB15	**OPJ19**	**S88**
950	600	900
760	540	750
600	480	400
500	300	340
280		250

Primer	**2a**	**12a**	**FS**
ISJ 4	44	44	-
	76	76	-
	132	132	132
ISJ 10	70	70	-
	110	110	-
	132	132	132
	166	166	-
	175	175	-
	187	187	-

Table 4.5. Microsatellite results for 13 primers. FS: field sample.

	Sample		
Locus (repeat motif)	***2a***	***12a***	***FS***
1 (CA)	244-254	244-254	244-254
3 (CA)	169	169	169
5 (GT)	**192-198**	**192-198**	**188-192**
9 (CT)	**159-174**	**159-174**	**169-184**
10 (GA)	233	233	233
14 (AG)	**228**	**228**	**214**
17 (AAG)	**159**	**159**	**162**
19 (AAG)	246-267	246-267	246-267
20 (TTC)	264-289	264-289	264-289
22 (GAT)	99-102	99-102	99-102
28 (AC)	**225-237**	**225-235**	**225-235**
29 (AAG)	194-197	194-197	194-197
30 (GAT)	139-142	139-142	139-142

General discussion

Biomonitoring has been proved as a reliable and cost-effective approach to control the air quality. There is indeed a long tradition using terrestrial mosses, but the comparison between the outcomes is at least complicated, as a result of the wide variability in the used methodologies.

The development and application of a novel, sustainable and standard biomonitor is the main objective of the Mossclone project. The experiments related to this project, even from a peripheral point of view, are useful to improve the current technologies or even for the development of new ones. For instance, the increment of biomass in *Rhynchostegium riparioides* opens a new horizon: the development of a novel and standard method to control the contamination of fresh water systems. Even if the growth was remarkably increased, further research is needed in order to: i) scale-up the production in photobioreactors, optimizing the operating parameters to increase the efficience of the process; and ii) further understand the development of new branches for the described rhyzoid structures, which undoubtely will lead to increase the biomass production.

The protoplast isolation reaction in *Sphagnum palustre* is another key issue. Protoplasts represent indeed, the starting point for all plant cell transformation protocols; and it could be desirable to joint moss clone growth for biomonitoring surveys to the production of biochemical products of biological/commercial interest produced in the enginerized moss clone. However, bryophyte cell walls, even if they lack lignin, present other recalcitrant compounds that render the digestion by hydrolitic enzymes highly difficult (however, see Batra *et al.*, 2003). The number of obtained protoplasts was remotely far away from other plant species, but still this is the first report of positive results in this field for this species. Further research is needed towards the improvement of the pre-cultivation protocols, or even better, for the establishment of "preserved-in-time" cultures of protonema. As a matter of fact, the possibility of establishment of a protonema culture altogether with an efficient protoplast isolation protocol could potentially guide to a new biotechnological perspective for *Sphagnum sp.* Furthermore, the availability of the whole sequence of this genus is nearby, being the use of this moss as a basic model for early plant evolution, similarly to *Physcomitrella patens*, a potentially forthcoming panorama.

The standardization of a biological material implies the achievement of the homogenization, from the morphological, chemical and genetic point of view. The main objective of this thesis was to characterize the *Sphagnum palustre* clone through the use of

DNA molecular markers. The molecular characterization concept is subject of diverse interpretations. It can be referred to whole organisms (e.g. for diagnosis purposes in commercial crops) or to specific genes. In the first case it is desirable to develop methodologies in order to distinguish the target organism of interest from other related organisms or even between different strains. Molecular markers are indeed frequently the only valid approach for this purpose. There is available a wide set of techniques in order to identify the desired individuals, which can have introduced and / or expressed a character of interest. For instance, higher production in crops or higher yield in some processes (see as an example Arif *et al.*, 2010, in desert plants) are conventionally ambitious goals.

Even if the worth of molecular markers applied to a wide range of fields (e.g. taxonomy, phylogeography, population genetics, molecular ecology), there were no reviews focused on the utilization of DNA molecular markers in mosses. Although a great deal of bibliography is available for vascular plants, molecular markers do not show same behaviour in bryophytes. An enlightening example comes from the barcoding sequences. Some of the proposed barcodes for flowering plants can not be applied in mosses. Therefore, at least a set of barcodes is necessary to characterize the bryophyte biodiversity (Liu *et al.*, 2010).

One of the main contributions of the published review by Crespo and co-workers (2014) is to provide an assessment of the current application of the techniques in bryophytes. Besides it is proposed as an useful first insight for bryologists into the PCR molecular performances in mosses. The construction of a summary table provides to the researchers a set of 108 molecular studies, classificated according to the field of study (biogeography, gene expression, phylogeny, population ecology, taxonomy and methodological-technical studies). Moreover this table provides the key words, in order to orientate the focus of the studies, the used technique and the considered taxa. In conclusion, this can be an useful tool for bryologists to choose the most suitable PCR technique for their particular aims.

Molecular phylogenetics has been extremely powerful for revisiting traditional taxonomic hypotheses, and this is especially true in taxa with reduced morphologies like bryophytes (Vanderpoorten & Shaw, 2010). The usefulness of the application of molecular markers in order to clarify phylogenetic relationships in mosses has been asserted since the end of '90s (Goffinet *et al.*, 1998). Absolutely the choice of loci is a critical issue. It is well known that nuclear loci are typically more variable than plastid loci, and nucleotide sequence data from one or a few loci are often insufficient at the species level. Sometimes

other types of molecular data (including RFLPs, ISSRs, and microsatellites) are especially useful for many species-level systematic problems (even if the biological species concept is still object of an ongoing debate).

The *Hypnum cupressiforme* complex (HCC) case constitutes a paradigmatic issue in systematics at specific level. Terracciano and co-workers (2012) have studied the HCC by the analysis of the intergenic spacers ITS1 and ITS2 of the nrDNA and chloroplast *trn*L_{UAA} intron. The results clearly highlight the presence of two well distinct taxa, *H. imponens* and *H. jutlandicum*. However, the circumscription of *H. lacunosum* was not clear, and therefore, the use of a multilocus technique, with higher resolution power (i.e. the ISJ markers) was justified.

As demonstrated in the results, it was possible the distinction between *H. cupressiforme* and *H. lacunosum*. Nevertheless, considering the low number of studied specimens, further research is clearly needed.

The main objective of the present PhD thesis was focused in the molecular characterization of the developed *Sphagnum palustre* clone by the Mossclone project consortium. The study of the *Sphagnum palustre* clone has required the use of both unilocus (i.e. SSR, sequencing and RFLP among others) and multilocus techniques.

Indeed *Sphagnum* occupies the most basal position in moss phylogenies; accordingly, sequences for the internal transcribed spacers of the nuclear ribosomal DNA can be easily aligned across its species (e.g. Shaw *et al.*, 2003). Furthermore, microsatellite primers developed for one group of closely related *Sphagnum* species, amplify homologous loci across the whole genus (Shaw *et al.*, 2008). By contrast, systematic studies *sensu lato*, mainly carried out by microsatellites, highlight the occurrence of interspecific hybridization, molecular divergence between disjunct populations, cryptic speciation and introgression, all phenomena demonstrating an ongoing molecular evolution in peat mosses, in striking contrast with the traditional idea of living fossils, frequently used to tag these plants. According, we found no polymorphisms between the moss clone and conspecific field samples in expressed sequences, but 2 SNPs were detected in the intron sequence analysed. Similarly, the analyses carried out by RFLP-PCR showed polymorphisms only in digested anonymous sequences, despite the high number of nuclear and plastid genes and spacers tested. All these considerations justify the use of both high and low resolution-power techniques for clone characterizations.

General conclusions

1. The biomass production in *Rhynchostegium riparioides* was remarkably increased. Further research is needed regarding the formation of branches from the described rhyzoid structures.
2. It was possible to extract a low number of protoplasts from *Sphagnum palustre* gametophytes. Further research is needed in order to improve and standardize the precultivation methods prior to the protoplast isolation reaction, and perform some trials of establishment of protonema cultures.
3. The state of the art regarding the PCR molecular markers in mosses was reviewed and published.
4. The first molecular technique that potentially could distinguish between *Hypnum cupressiforme* and *H. lacunosum* was developed.
5. The *Sphagnum palustre* clone was accurately characterized through the use of DNA molecular markers.

Publications and contributions to congress

Crespo D, Terracciano S, Giordano S & Spagnuolo V (2014) Molecular markers based on PCR methods: a guideline for mosses. Cryptogamie 35(3): 229-246.

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Manuscripts in preparation

Molecular and chemical characterization of a moss clone (*Sphagnum palustre*) for its use as an active biomonitor.

Development of novel markers for the genus *Sphagnum*.

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Annex

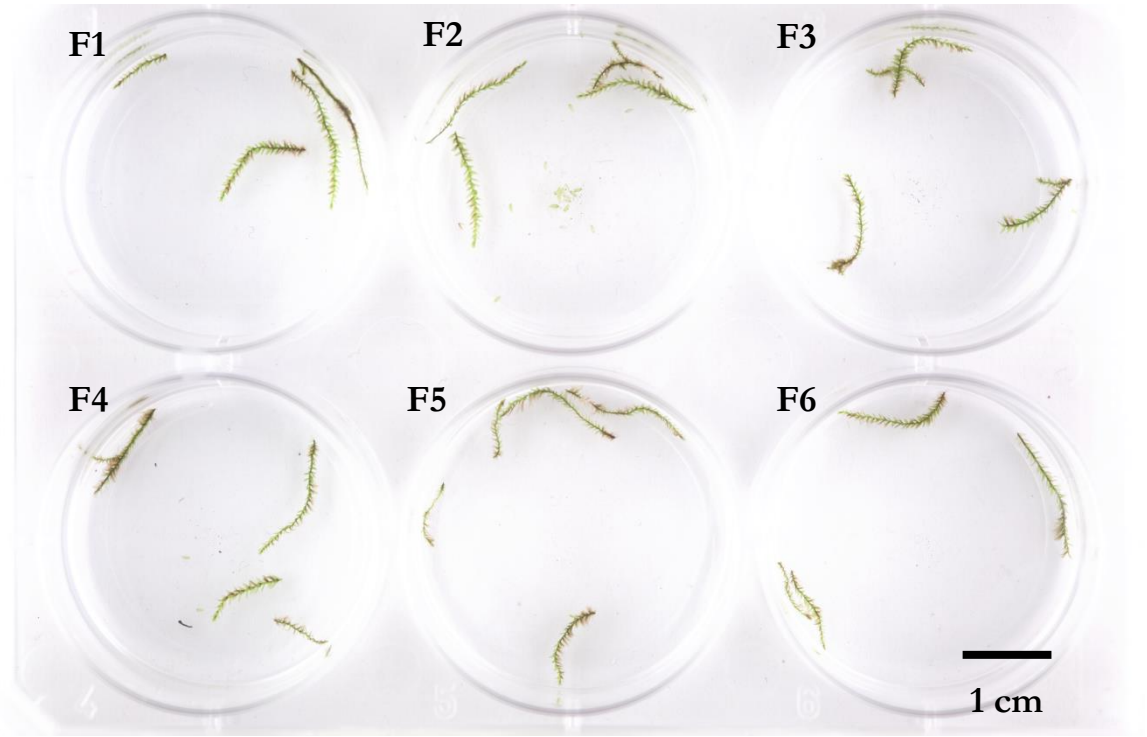


Figure A1. General appearance of *Rhynchostegium riparioides* gametophytes prior to the experiment 2.

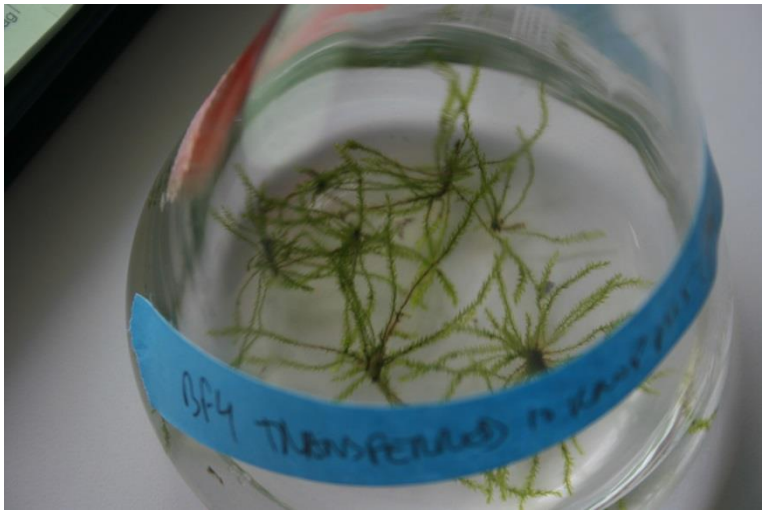


Figure A2. Multiple branching sites. Notice the multiple pattern of development of branches from the brownish central points, full of rhyzoids.

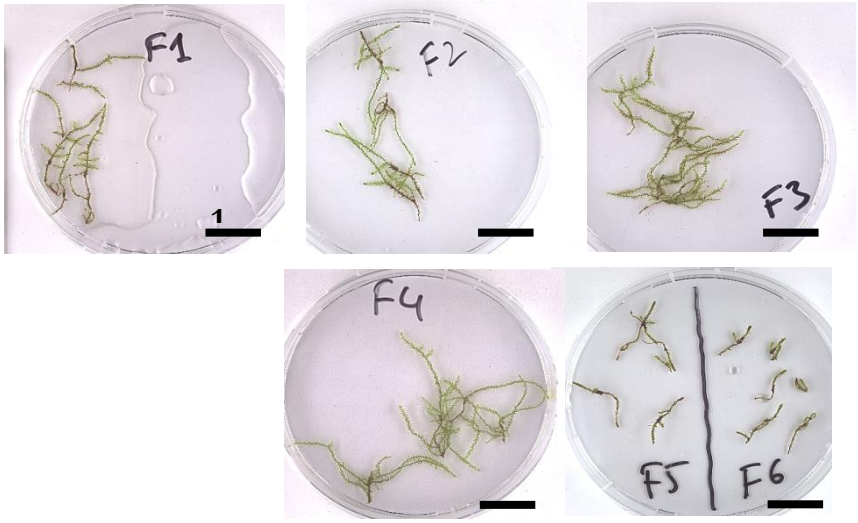


Figure A3. General appearance of *Rhynchostegium riparioides* gametophytes after the experiment 2.

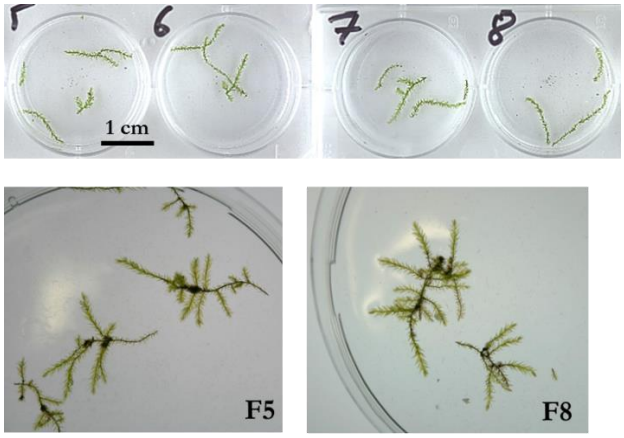


Figure A4. Initial and final appearance of *Rhynchostegium riparioides* gametophytes prior and after the experiment 3.

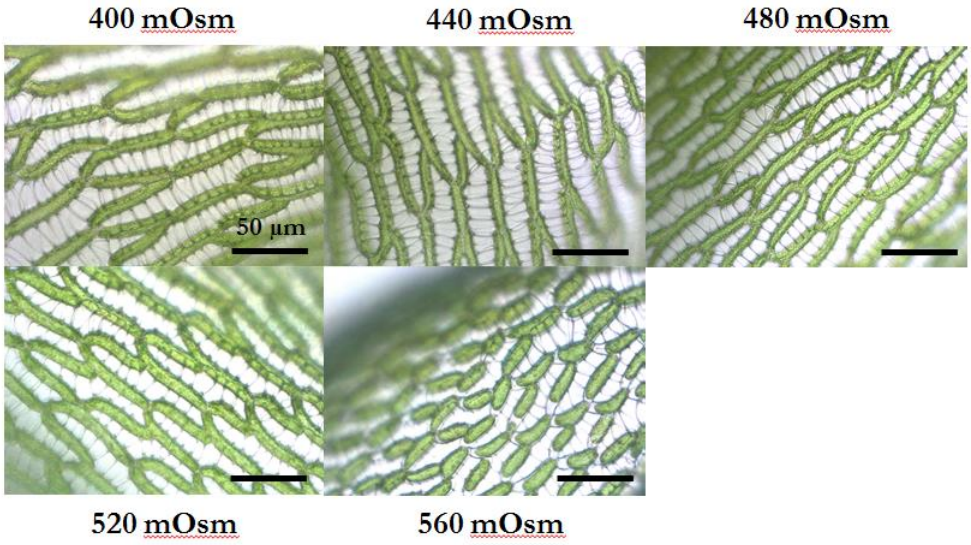


Figure A5. Osmolarity (mOsm) adjustment with mannitol in leaves of *Sphagnum palustre*.

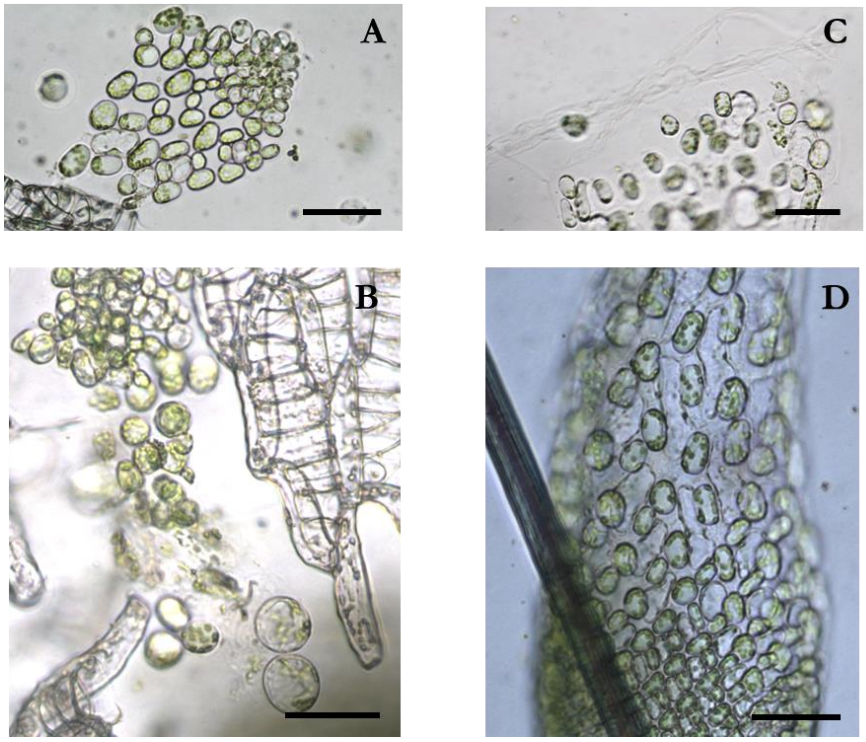


Figure A6. Adjustment of the osmolarity directly in the protoplast isolation reactions. A: 320 mOsm; B: 450 mOsm; C: 475 mOsm; D: detail of the release of the protoplasts from a leave of *Sphagnum palustre*.

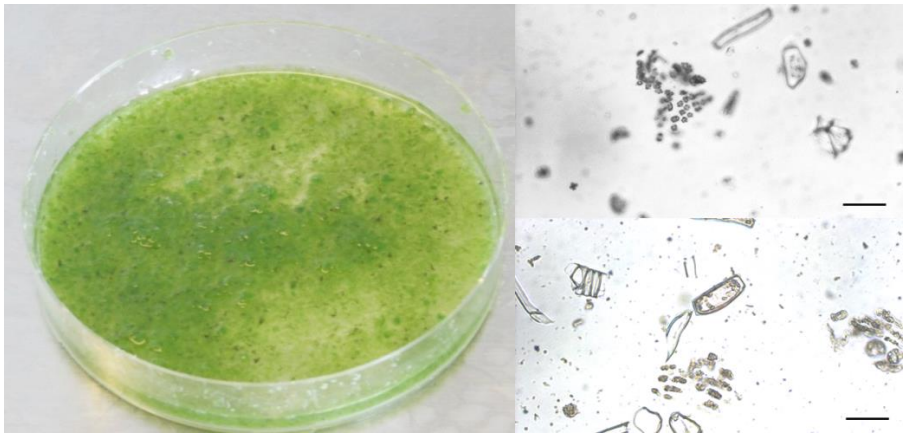


Figure A7. Detail of the degree of disruption of the gametophytes of *Sphagnum palustre* previous to the digestion with driselase.Figure A8. Dead protoplasts of *Sphagnum palustre*after a digestion with driselase set at 37ºC. Scale: 50 µm.

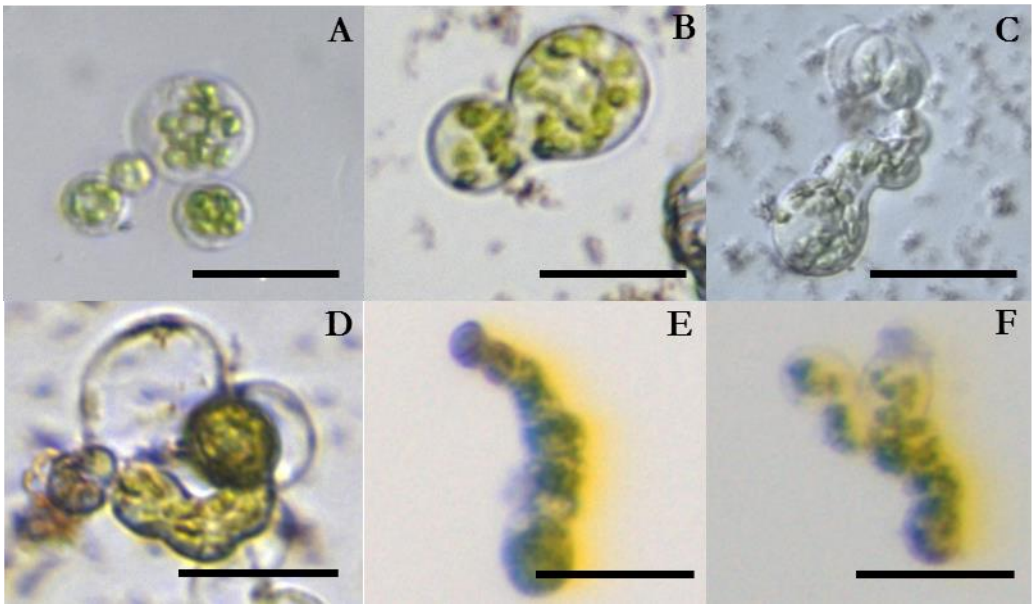
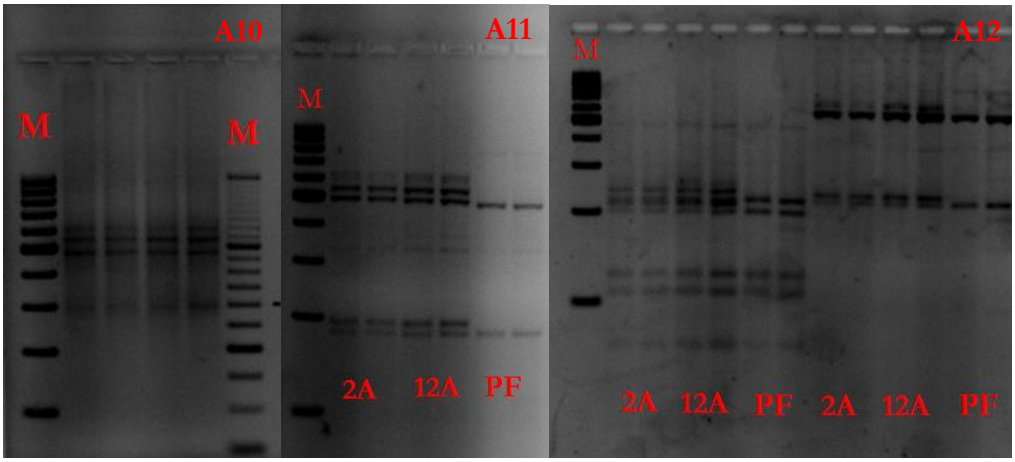


Figure A9. Regeneration stages in *Sphagnum palustre* protoplasts. Scale: A, B, C, D: 50 µm; E, F: 100 µm.



Figures A10, A11 and A12. A10: RAPD profile for the primer OPJ19 in the *Sphagnum palustre*clone. A11: Double digestion of the RAPDf sequenceamplificated in *Sphagnum palustre* clone with *Hind*III/*Hinf*I. A12: Single digestion of RAPDf sequence of *Sphagnum palustre* clone with *Mnl*I (first 6 samples) and *Eco*RI.M: GeneRuler 1 kb and 500 bp (only in A10) DNA Ladder (Thermo Scientific).

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