A novel molecular protocol for the rapid extraction of DNA from bryophytes and the utility of direct amplification of DNA from a single dwarf male

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Abstract. A novel protocol for the rapid extraction of bryophyte DNA is presented and tested on nine mosses and one liverwort. Amplification products and sequences of the *rps*4 gene were obtained for all the samples tested. Direct amplification and sequencing of DNA from a single dwarf male was found to be possible. By adding single dwarf males of *Dicranum scoparium* directly to a PCR, amplification products of the ITS regions were obtained for nine of the 11 dwarf males tested. To obtain different gene sequences from a single dwarf male, individual dwarf males were incubated in buffer at 60°C for different time periods and the resulting suspensions used for amplification of the chloroplast regions *trn*G and *trn*L-F. Amplification products of the *trn*G region were obtained for all the samples, but amplification of the *trn*L-F region was less successful. Clean DNA sequences were obtained from all the amplification products that were used in bi-directional sequencing. The rapid method presented has the potential to be a useful tool for screening high numbers of plants for specific genomic markers, such as in DNA barcoding. Direct amplification of DNA provides the opportunity for the first time to study genetic variation among moss dwarf males.

Keywords. Dwarf males, rapid extraction, direct PCR, phyllodioicy, *Dicranum*, *Garovaglia*, Ptychomniaceae.

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Conventional protocols used for extraction of plant DNA typically follow those of Doyle and Doyle (1987, 1990). These protocols are time-consuming and involve the use of potentially toxic chemical compounds such as CTAB, chloroform and β-mercaptoethanol, and sometimes significant amounts of plant material. Alternative methods for rapid preparation of plant tissue for PCR have been described by Berthomieu and Meyer (1991) and Rogers and Parkes (1999), who both employed direct amplification of DNA from plant tissue, and Wang et al. (1993), who used NaOH to isolate genomic DNA. Recently, Werner et al. (2002) successfully employed these techniques on 15 mosses and two liverworts. Rapid techniques are particularly useful when a large number of specimens have to be processed, for example in DNA barcoding (Kress et al. 2005), where short DNA sequences are used for the rapid and unequivocal identification of species, but also for extremely small bryophytes when conventional extraction protocols are impossible.

Rapid techniques could be especially useful for

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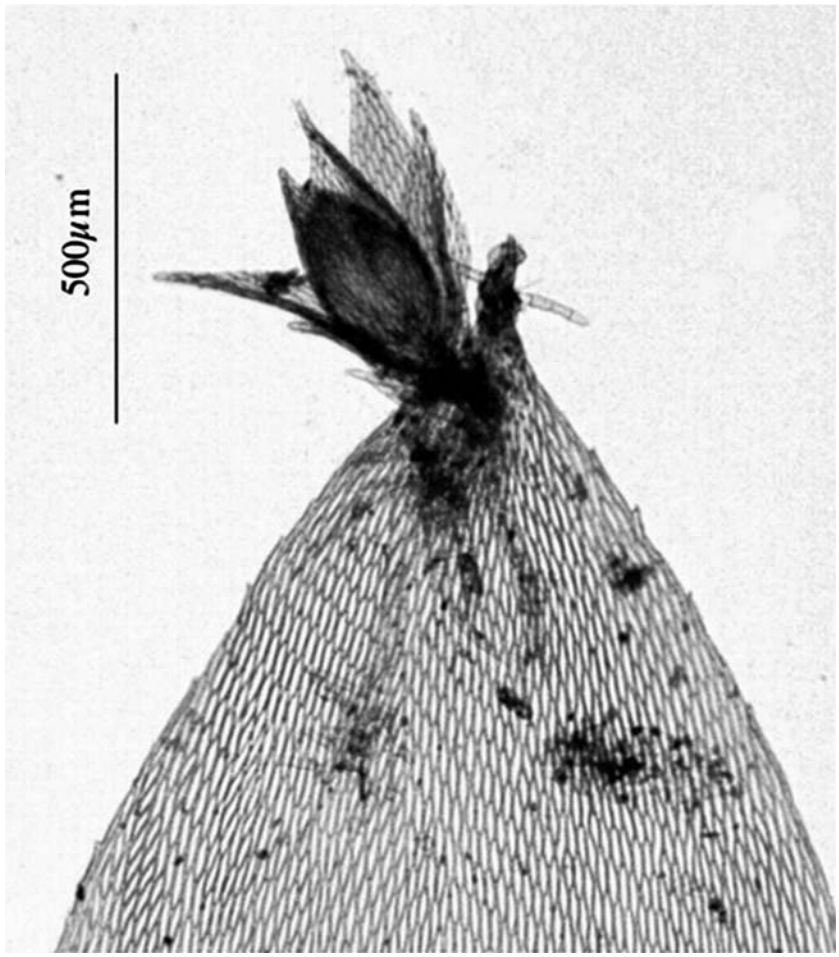

Figure 1. Dwarf male of *Garovaglia elegans* attached to the leaf apex of its female host.

exploring genetic variation among and within phyllodioicous taxa. Phyllodioicy is a reproductive strategy within mosses where dwarf males germinate from spores on female plants. These spores may be derived from sporophytes on the host plant or sporophytes of nearby or distant populations of the same species. Genetic variation and the source of the dwarf males could be explored by sequencing individual dwarf males colonizing a single female or population of female plants, and comparing sequences with those obtained from the potential sources of the spores. However, given the small size (<1 mm) of the dwarf males (**Fig. 1**) the amount of DNA that can be extracted using conventional techniques is inadequate, and alternative protocols need to be developed.

In this paper, we describe a protocol for the rapid extraction of genomic DNA from bryophytes in general. We also show the utility of direct amplification methods for the generation of DNA sequences from a single dwarf male.

Material and Methods

Sampling of plant material and DNA. Nine mosses and one liverwort were sampled for the rapid extraction of genomic DNA, namely, *Amphidium mougeotii* (Bruch & Schimp.) Schimp., *Breutelia chrysocoma* (Hedw.) Lindb., *Bryum bicolor* Dicks., *Ctenidium molluscum* (Hedw.) Mitt., *Dicranum scoparium* Hedw., *Garovaglia elegans* Müll. Hal., *Garovaglia powellii* Hampe, *Homalothecium sericeum* (Hedw.) Schimp., *Rhytidiadelphus squarrosus* (Hedw.) Warnst. and *Herbertus aduncus* (Dicks.) Gray. Dwarf males were sampled with forceps under the dissecting

microscope from recent field collections of *D. scoparium*, *G. elegans* and *G. powellii*. In *D. scoparium,* the dwarf males are nested among the tomentum along the stem, whereas in *G. elegans* and *G. powellii* they are situated on the leaves of their female host (**Fig. 1**). In addition, DNA was extracted from shoot and leaf apices of a female plant of *D. scoparium* and its seta, using the conventional extraction protocol of Doyle and Doyle (1987), modified as in Shaw (2000). The taxa and individual dwarf males included in this study are listed in **Table 1** with GenBank accession numbers and voucher information.

For the rapid extraction method, sequences were obtained for the chloroplast gene *rps*4. For the direct PCR methods, sequences were obtained for two chloroplast genomic regions, namely, the *trn*L (UAA) 5' exon – *trn*F (GAA) region (*trn*L-F) and the *trn*G (UCC) intron (*trn*G), plus the nuclear ITS1 – 5.8S rRNA – ITS2 region (ITS).

Molecular protocols. For the rapid extraction of DNA, small fragments (<10 mg) of shoot and leaf apices were placed into a 1.5 ml Eppendorf tube. Liquid nitrogen and sterile sand were added and immediately after the nitrogen had sublimed, the sample was ground with a micropestle, diluted in 50 µl 100 mM TE-buffer, and incubated at 60°C for 15 min. The suspension was cleared by centrifugation at 13,200 rpm for 10 min, the supernatant transferred to a clean 1.5 ml Eppendorf tube, and 2 µl was immediately used for PCR. The remaining sample was stored at −20°C for 28 days. The PCR was repeated on the stored sample to test for the stability of the DNA.

For amplification of the ITS regions, eleven dwarf males (≪1 mg) were sampled from a female shoot of *Dicranum scoparium* and added directly to PCR reactions. The main problem with this approach is that the dwarf males cannot be reused for different gene studies so only a single gene can be studied for each individual. We therefore conducted an experiment to explore the feasibility of extracting DNA for use in multiple PCR reactions by incubation of individual dwarf males in 100 mM TE-buffer. Four treatments were used to determine optimal incubation time. A total of 112 dwarf males from three species (*D. scoparium* (n = 60), *G. elegans* (n = 20), and *G. powellii* (n = 32)) were used in the four treatments. The individual dwarf males were placed in 0.5 ml PCR tubes and 10 µl 100 mM TE-buffer was added to each of the tubes. The four treatments were incubated at 60°C for 15, 30, 45 and 60 minutes, respectively, and 5 µl of the suspension was then transferred to PCR tubes containing reagents for amplification of the *trn*L-F and *trn*G regions, respectively. Amplification products obtained from the most successful treatment were used in bi-directional sequencing.

PCR reactions were prepared using 2.5 units of *Taq* polymerase (Bioline) in a total of 25 µl reaction volume (1 × thermostable buffer, 2.5 mM $MgCl_2$, 100 µM dNTPs, 100 µM primer). For amplification of the ITS regions, betaine (1.2 M; Sigma-Aldrich) was added to prevent formation of secondary structures. Double-stranded DNA templates were prepared with 30 cycles, preceded by an initial melting step of 5 min at 94°C and followed by a final extension period of 7 min at 72°C. For each genomic region, PCR was optimized as follows: *rps*4 and *trn*L-F—94°C (30 sec), 50°C (30 sec), 72°C (1 min); *trn*G—94°C (30 sec), 52°C (30 sec), 72°C (1 min); and ITS—94°C (30 sec), 50°C (30 sec), 72°C (2 min). The primer sets trnC and trnF (Taberlet et al. 1991) and rps5 (Nadot et al. 1994) and trnas (Cox et al. 2000) were used for amplification of the *trn*L-F region and the *rps*4 gene, respectively. Amplification products for the *trn*G region were accomplished using the primer combination trnGF and trnGR (Pacak & Szweykowska-Kulińska 2000). The ITS regions were amplified using primers AB101 and AB102 (Douzery et al. 1999).

Amplified fragments were cleaned using the GFX PCR DNA purification kit (Amersham Biosciences, Little Chalfont, UK) and eluted in 30 µl nanopure water. Bi-directional sequencing for each gene was performed using each amplification primer in conjunction with the ABI BigDye DiDeoxy Terminator Cycle Sequencing Reaction Kit following manufacturer's instructions on an ABI PRISM 377 automated sequencing machine.

Sequence manipulation and alignment. Nucleotide sequences were edited and assembled with Sequencher 4.5 (Genes Code Corporation). The assembled sequences were aligned manually using Se-Al v 2.0 (http://evolve.zoo.ox.ac.uk/software.html?id=seal) and BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) were conducted to confirm the identity of the generated sequences.

Table 1. Taxa and dwarf males included in the current study with GenBank accession numbers and voucher information. Year of collection of voucher is given for the taxa of which sequences were generated in this study. Herbarium acronyms follow Holmgren and Holmgren (http://sciweb.nybg.org/science2/IndexHerbariorum.asp). **A.** Rapid extraction methods. **B.** Direct amplification of dwarf males. For vouchers with more than one dwarf male sampled, some accession numbers are sequential and only the smallest and largest numbers are given. Sequences not generated in this study are in bold. Asterisks (*) indicate that DNA was extracted with the conventional extraction protocol of Doyle and Doyle (1987), modified as in Shaw (2000). [1] Leaf tips of a female plant; [2] Seta.

A. Rapid extraction

Taxon	Voucher information	Year of collection	*rps*4
Amphidium mougeotii	*Pedersen 16/04* (BM)	2004	DQ294316
Breutelia chrysocoma	*Pedersen 15/04* (BM)	2004	DQ294321
Bryum bicolor	*Pedersen 7/04* (BM)	2004	DQ294323
Ctenidium molluscum	*Pedersen 12/04* (BM)	2004	DQ294320
Dicranum scoparium	*Newton 6829* (BM)	2005	DQ294317
Garovaglia elegans	*Newton 6560* (BM)	2003	DQ294318
Garovaglia powellii	*Newton 6496* (BM)	2003	DQ296008
Homalothecium sericeum	*Pedersen 10/04* (BM)	2004	DQ294319
Rhytidiadelphus squarrosus	*Pedersen 2/04* (BM)	2004	DQ294322
Herbertus aduncus	*Pedersen 14/04* (BM)	2004	DQ294324

B. Direct amplification of dwarf males

Taxon	Voucher information	Year of collection	*trn*G	*trn*L-F	ITS
Dicranum scoparium	*Newton 6829* (BM) [1*]	2005			DQ294335
	Newton 6829 (BM) [2*]	2005			DQ294334
	Newton 6829 (BM)	2005	DQ295845–DQ295859	DQ294336–DQ294343	DQ294325–DQ294333
Euptychium cuspidatum	*Newton 5373* (BM)		**DQ194233**	**DQ194209**	
Euptychium dumosum	*Crosby 14280* (NY)		**DQ194234**	**DQ194210**	
Euptychium mucronatum	*Streimann 50150* (NY)		**DQ194235**	**DQ194211**	
Euptychium robustum	*Streimann 56055* (CBG), *56137* (NY)		**DQ194236**	**AY306741**	
Euptychium setigerum	*Crosby 81325* (CBG)		**DQ194237**	**DQ194212**	
Euptychium vitiense	*Buck 7255* (NY)		**DQ194238**	**AY306743**	
Garovaglia angustifolia	*Stephen et al. VN SBC6351* (SINU)		**DQ194239**	DQ194213	
Garovaglia baeuerlenii	*Schumm/Schwarz 4719* (SINU)		**DQ194240**	**DQ194214**	
Garovaglia binsteadii	*Iserentant n B-73* (NY)		**DQ194241**	**AY306747**	
Garovaglia compressa	*Raymod et al. VN SBC6376 (SINU) / Akiyama C-16340* (NY)		**DQ194242**	**AY306748**	
Garovaglia elegans	*Newton 5465* (BM) *		**DQ194243**	**DQ194215**	
	Newton 6524 (BM)	2003	DQ296009, DQ296011–DQ296013	DQ296022–DQ296024	
	Newton 6560 (BM)	2003	DQ296010		
Garovaglia plicata	*Ellis BF9512* (BM)		**DQ194244**	**DQ194216**	
Garovaglia powellii	*Newton 6496* (BM) *		**DQ194245**	**DQ194217**	
	Newton 6461 (BM)	2003	DQ296014-DQ296016, DQ296021	DQ296025-DQ296027	
	Newton 6552 (BM)	2003	DQ296017	DQ296029	
	Newton 6496 (BM)	2003	DQ296018	DQ296028	
	Newton 6474 (BM)	2003	DQ296019		
	Newton 6442 (BM)	2003	DQ296020		

Table 1. Continued.

B. Direct amplification of dwarf males

Taxon	Voucher information	Year of collection	*trn*G	*trn*L-F	ITS
Garovaglia subelegans	*Sloover 43.136* (BM)		**DQ194246**	**DQ194218**	
Garovaglia zantenii	*Norris 65427* (BM)		**DQ194247**	**DQ194219**	
Glyphothecium sciuroides	*Bell 542* (BM)		**DQ194249**	**DQ194221**	
Ptychomnion aciculare	*Newton 5489* (BM)		**DQ194254**	**DQ194225**	

For *Garovaglia elegans* and *G. powellii,* the *trn*L-F and *trn*G sequences obtained from the dwarf males were aligned with sequences of female plants. *Trn*L-F and *trn*G sequences of other species of *Garovaglia* and species of the genus *Euptychium,* which according to Pedersen and Newton (2006) is strongly supported as sister to *Garovaglia,* were also included (**Table 1**). In addition, the alignments included sequences of *Glyphothecium sciuroides* (Hook.) Broth. and *Ptychomnion aciculare* (Brid.) Mitt. Regions of incomplete data and ambiguous alignment were identified and excluded from subsequent analyses. The resulting alignment, hereinafter dwarf male data set, was exported as a NEXUS file.

Phylogenetic analyses. To explore the phylogenetic affinity of dwarf males of *G. elegans* and *G. powellii*, we conducted maximum parsimony analyses of the dwarf male data set using PAUP4.10b (Swofford 2002), with *Glyphothecium sciuroides* and *Ptychomnion aciculare* as outgroup taxa. The analyses were performed including only parsimony informative characters. Heuristic searches were conducted with 10,000 replicates of random addition sequence and TBR branch swapping. Bootstrap support values for branches were estimated by 300 replicates of one random addition sequence.

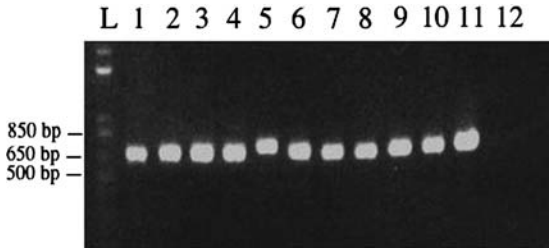

Figure 2. Amplification of the *rps*4 gene using DNA extracted with the rapid extraction protocol. L = 1 kb plus ladder (Invitrogen Life Technologies); 1 = *Amphidium mougeotii;* 2 = *Breutelia chrysocoma;* 3 = *Dicranum scoparium;* 4 = *Garovaglia powellii;* 5 = *Herbertus aduncus;* 6 = *Garovaglia elegans;* 7 = *Bryum bicolor;* 8 = *Ctenidium molluscum;* 9 = *Rhytidiadelphus squarrosus;* 10 = *Homalothecium sericeum;* 11 = Positive control; 12 = Negative control.

Results

Rapid extraction method. For the *rps*4 gene, amplification products (**Fig. 2**) and sequences were obtained for all the studied species. All bands were reproduced when the PCR was repeated after 28 days of storage at −20°C (results not shown). While this indicates that there is no short-term degradation of DNA, it will be necessary to further investigate this by conducting additional amplifications of long-term stored samples and to screen other genomic regions. The BLAST searches confirmed the identity of the obtained *rps*4 sequences of *Dicranum scoparium*, *Garovaglia elegans*, *Breutelia chrysocoma*, *Rhytidiadelphus squarrosus* and *Bryum bicolor*. No *rps*4 sequences of the remaining species were available in GenBank but for these species the results of the BLAST searches produced significant alignments with *rps*4 sequences from congeneric species (e.g., *Amphidium lapponicum* for BLAST search of the *rps*4 sequence of *A. mougeotii*).

Direct amplification of dwarf males. When individual dwarf males of *Dicranum scoparium* were added directly to PCR reactions, amplification and sequencing of the ITS regions was successful for nine of the 11 dwarf males (**Fig. 3**). Alignment of these ITS sequences revealed strong similarity among dwarf males, the female plant, and its seta, except for a T-to-C substitution in the ITS1 region of one dwarf male and a one base insertion in the ITS2 region of four dwarf males (**Fig. 4**).

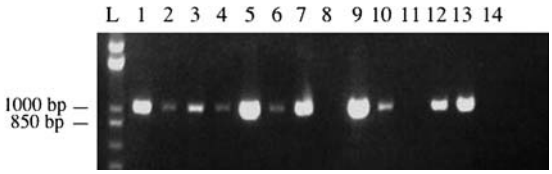

Figure 3. Amplification of the 18S ITS1 – 5.8S rRNA – ITS2 region with the direct PCR method for dwarf males of *Dicranum scoparium*. L = 1 kb plus ladder (Invitrogen Life Technologies); 1–11 = dwarf males; 12 = *D. scoparium* female shoot; 13 = *D. scoparium* seta; 14 = negative control.

When single dwarf males were incubated and the suspension subsequently used for PCR, amplification products of the *trn*G region were obtained for all samples of each replicate (results not shown). By contrast, PCR of the *trn*L-F region was less successful with a maximum of eight, three and five amplification products obtained for *Dicranum scoparium*, *Garovaglia elegans* and *G. powellii,* respectively, after an incubation period of 30 min. Given the small size of the dwarf males, a low template concentration or copy number, rather than the length of the incubation period, is probably the limiting factor. Nevertheless, sequences were generated from all the amplification products obtained from samples incubated for 30 min. Within each species, alignment of each of the two chloroplast regions showed almost complete similarity between dwarf males and BLAST searches confirmed the identity of the sequences.

Phylogenetic analyses. Alignment of the dwarf male data set resulted in 1,132 nucleotide sites (475 *trn*L-F and 657 *trn*G), of which 353 were excluded due to missing data and regions of ambiguous alignment. Of the 779 included nucleotide sites, 57 were parsimony informative (17 *trn*L-F and 40 *trn*G).

The maximum parsimony analysis resulted in four most parsimonious trees of the length 93, consistency index (CI) 0.669, and retention index (RI) 0.900. One of the most parsimonious trees with bootstrap support values is presented in **Fig. 5**. The dwarf males and the female plant of *Garovaglia elegans* form a monophyletic group but the clade is unresolved and poorly supported (BS = 59). The dwarf males and the female plant of *G. powellii* form a robust clade (BS = 99) but the phylogenetic relationships within the clade are mostly unresolved. However, it is noteworthy that one of the dwarf males of *G. powellii* sampled from the female plant with accession number 6461 is more closely related to a dwarf male from another female plant (accession number 6552) than to any of the other two dwarf males from accession 6461, but this inference is poorly supported (BS = 63). These results suggest that the dwarf males of the two *Garovaglia* species are conspecific with their respective female host, but further sampling of dwarf males from additional field collections is needed to confirm this.

Discussion

Even though rapid extraction protocols provide considerable savings in time and resources compared to conventional extraction protocols they have rarely been used for bryophytes. Generally, DNA obtained with conventional extraction protocols is cleaner and less susceptible to degradation, but rapid extraction protocols may nonetheless be useful when screening

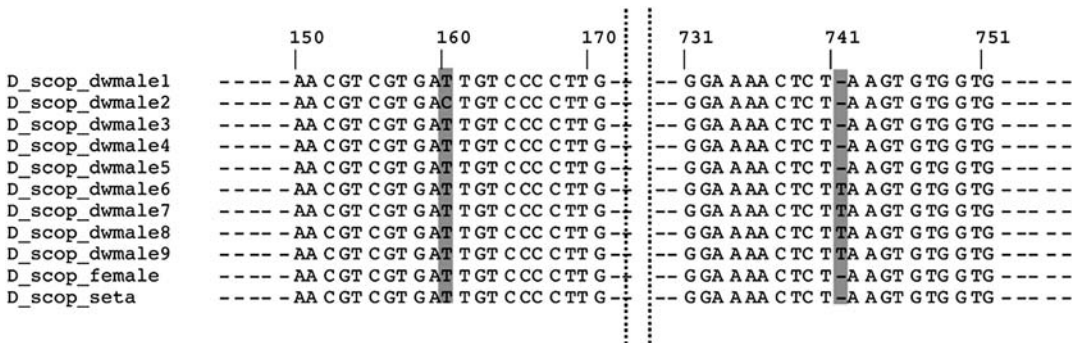

Figure 4. Part of the alignment of the ITS1 – 5.8S – ITS2 region for nine dwarf males plus a female plant and its seta of *Dicranum scoparium*. Shaded areas indicate a T-to-C substitution at nucleotide position 160 of dwarf male #2 and an insertion in four dwarf males at position 741, respectively.

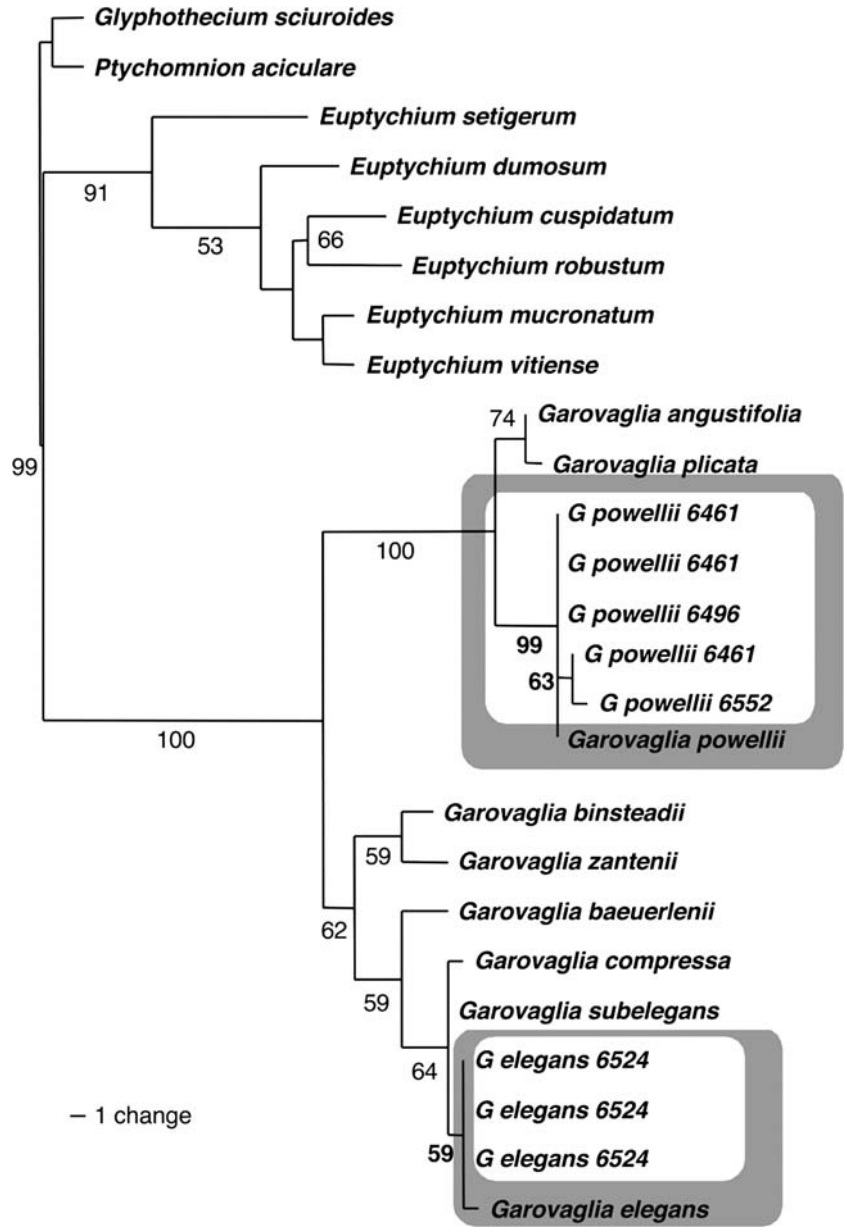

Figure 5. Phylogram of one of the four most parsimonious trees obtained from the maximum parsimony analysis (length 93, CI 0.669, RI 0.900) with bootstrap support values (≥50%). Gray areas indicate the clades with *Garovaglia elegans* and *G. powellii*. Dwarf males are within white boxes.

plants for specific genomic markers, for instance when searching for genes suitable for DNA barcoding (Kress et al. 2005), or when large numbers of species are studied. One reason for the success of the rapid extraction method may be that the small amounts of plant tissue used may minimize the concentrations of enzymes, polysaccharides and other potential inhibitors of PCR. Likewise, low concentrations of degrading agents may make it possible to store the DNA for longer periods of time.

The most exciting result of this study is the possibility of direct amplification of individual dwarf males. Dwarf males are found in a wide variety of unrelated groups of mosses (Ramsay 1979) including

some groups of closely related species. However, the extreme reduction of many of the dwarf males makes it impossible to use morphology to confirm the identity of a single dwarf male. Furthermore, since the spores on a given female host may come from different sources, including the host plant or conspecific populations, one female could potentially host a variety of dwarf males. The results of this study indicate that there may be genetic variation in the ITS regions among dwarf males from a single female host. However, studies based on extensive sampling of female plants and their dwarf males are needed to explore the genetic diversity of dwarf males more in detail. The molecular protocols presented in this study provide tools ideally suited to exploring the genetic variation among dwarf males. Direct amplification of individual dwarf males makes it feasible to test this possibility, and will be an essential tool for studies of population biology and evolutionary processes in phyllodioicous plants.

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