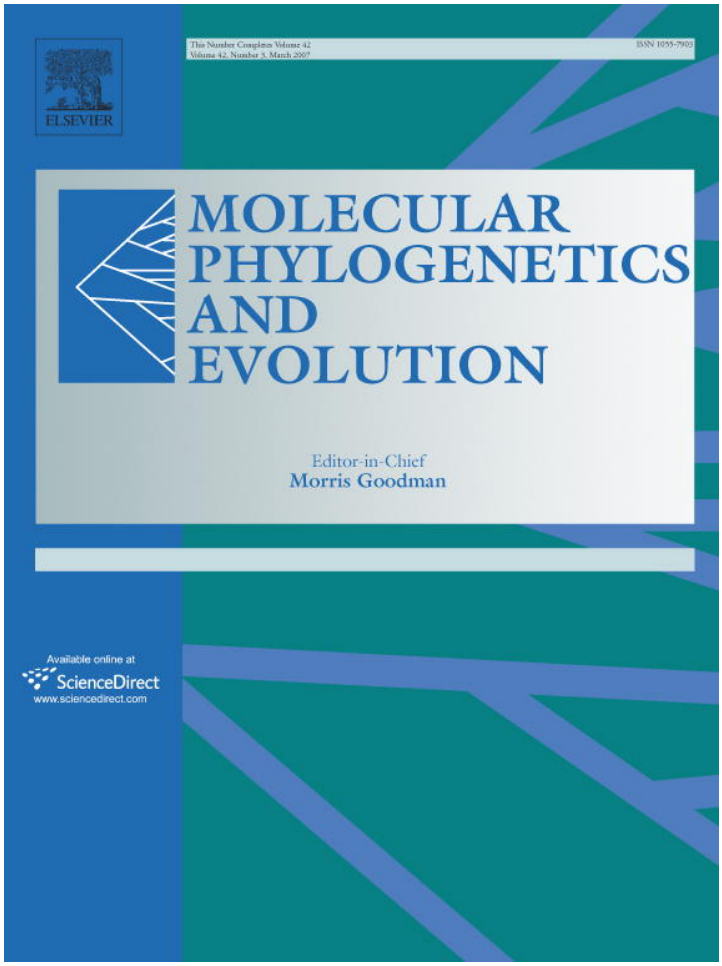

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Molecular and morphological incongruence in European species of *Isothecium* (Bryophyta)

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Abstract

During the identification of Moroccan samples a plant belonging to *Isothecium* with characteristics of *I. alopecuroides* (Dubois) Isov. and, to a smaller degree, *I. algarvicum* W.E. Nicholson and Dixon was found. Problems with attributing the plant to any of the European *Isothecium* species and the known large morphological variation in *I. alopecuroides* suggested that molecular studies were needed to evaluate patterns of relationships in this complex. We investigated one nuclear and one chloroplast marker from 66 samples (gametophytes) of *Isothecium alopecuroides* and from 18 samples of other *Isothecium* species. Parsimony and likelihood (via Bayesian analysis) were used as optimality criteria to compute phylogenetic trees. Bootstrapping and posterior probabilities were used, not only to quantify support, but also to evaluate competing phylogenetic alternatives in consensus networks. Finally, split decomposition and neighbour net analysis were used to compute distance based split networks, in order to avoid systematic error. The observed discrepancy among morphological and molecular data suggests that none of the European species *Isothecium alopecuroides*, *I. holtii* and *I. myosuroides* are monophyletic as defined by traditional morphological characters. Convergent morphological evolution cannot explain the discrepancy in this particular case; instead exchange of genetic material among *Isothecium* species is considered a potential explanation for the molecular diversity within morphospecies.

Keywords: Bryophytes; Phylogeny; Hybridization; ITS; trnG; Isothecium

1. Introduction

Isothecium Brid. is a genus of dioicous pleurocarpous mosses that belongs to the Lembophyllaceae s.l. (Huttunen et al., 2004). Its members can be easily differentiated from most other pleurocarpous mosses by their incrassate, usually partly bistratose, and opaque alar cells, which form a distinct yellowish to brownish group. In Europe, the genus is represented by *I. alopecuroides* (Dubois) Isov., *I. algarvicum* W.E. Nicholson and Dixon, *I. myosuroides* Brid. and *I. holtii* Kindb. (Corley et al., 1981). Morphological characters, such as stem leaf shape and margin denticulation, differentiate these four species, although a large morphological variation is observed in *I. alopecuroides* and *I. myosuroides*.

Isothecium holtii and *I. myosuroides* are both separated from *I. alopecuroides* by their stem leaf margins obscurely denticulate to more or less dentate from leaf base to apex, rather than only in upper leaf margin. The two species differ mainly in leaf shape; in *I. myosuroides* leaves taper to a narrow long or short acuminate apex, whereas in *I. holtii* leaves taper gradually or abruptly to an acute or broadly acuminate apex. In Europe the variety *I. myosuroides* var. *brachythecioides* (Dixon) C.E.O. Jensen is sometimes distinguished from *I. myosuroides* var. *myosuroides* by procumbent secondary stems (more or less erect in var. *myosuroides*), long, irregular and distant branches (vs. short and subdendroid ones) and by imbricate leaves when moist (erect-patent in var. *myosuroides*). However, it is linked to var. *myosuroides* by intermediates and is therefore not accepted by all

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authors (Smith, 2004). Also in North America a number of taxa close to *I. myosuroides* have been recognized (Ryall et al., 2005).

Both *Isothecium alopecuroides* and *I. algarvicum* have stem leaves shortly pointed and margins denticulate only towards leaf apex, but differ in their average size (larger in *I. alopecuroides*), leaf shape (obovate to sometimes ovate-oblong with obtuse to apiculate apex in *I. alopecuroides*, ovate to triangular-ovate with acute to acuminate apex in *I. algarvicum*), the supra-alar cells (more strongly differentiated from the surrounding cells and reaching further up along branch and stem leaf margins in *I. algarvicum*) and the capsules, more or less erect in *I. alopecuroides* and inclined to horizontal in *I. algarvicum* (Hedenäs, 1992).

During the identification of Moroccan samples, plants with gametophytic characteristics of *Isothecium alopecuroides* and, to a smaller degree, *I. algarvicum*, were found. They differ from the first by the smaller average size and by their supra-alar cells, which extend about 1/4 or sometimes more up along the branch leaf margins. As mentioned above, *I. algarvicum* also has such supra-alar cells, yet they appear not only in the branch leaves but also in the stem leaves, and this species has a different leaf shape. The Moroccan material was compared with numerous samples of *I. alopecuroides* in the Swedish Museum of Natural History (S). In this process, a great morphological variation was observed among the *I. alopecuroides* samples, especially in leaf shape and plant size. Complementary morphometrical studies were performed and showed some characters overlapping between the Moroccan material and that of studied *I. alopecuroides*. Nevertheless, the combination of characters exhibited by the Moroccan samples was unique. We first thought that the special Moroccan material could belong to *I. marocanum* Thér. and Meyl. but the type of this name belongs to *Scorpiurium deflexifolium* (Solms) M. Fleisch. and Loeske (Draper and Hedenäs, 2005). The difficulty of attributing the plant to any of the *Isothecium* taxa and the large morphological variation in *I. alopecuroides*, suggested that molecular studies were needed to evaluate patterns of relationships in this complex.

Specific and infraspecific level relationships among bryophytes have already been analysed by molecular methods at several occasions and including various markers (e.g., Chiang and Schaal, 1999; Shaw and Allen, 2000; Shaw, 2001; Shaw et al., 2002; Werner et al., 2002). Within *Isothecium*, relationships among species and morphotypes were analysed in the *I. myosuroides* complex in North America by Ryall et al. (2005). These authors studied nucleotide sequences from two chloroplast loci and concluded that there is a genetic basis for the differentiation of *I. myosuroides* and *I. stoloniferum*, as well as between the morphotypes of the latter, even though unambiguous morphological characters to support the division of these taxa have not yet been found. The present study aims to determine if there is also a molecular basis for the morphological variability within *Isothecium alopecuroides*, at the same time evaluating possible geographical differentiation. For this purpose, we investigate one nuclear marker (ITS) and the tRNA-Gly (trnG) of the chloroplast. It has been demonstrated that phylogenetic tree-building methods may be insufficient to describe fully evolutionary pathways and are susceptible to systematic error (Bryant and Moulton, 2004; Delsuc et al., 2005; Esser et al., 2004; Holland et al., 2004; Winkworth et al., 2005). Systematic error is likely to occur, if one considers large and heterogeneous data sets (such as in multi-gene analysis) that, in addition, might include possible reticulate phenomena (Huson and Bryant, 2006). In such cases, any phylogenetic tree-building method is strongly biased by the a priori assumptions causing reconstruction artefacts (Steele, 2005) and is strongly limited in its possibilities to adequately model the data and to provide a phylogenetic hypothesis (Huson and Bryant, 2006). As will be shown, these objections apply to the assembled ITS and tRNA-Gly data of our *Isothecium* species.

2. Materials and methods

2.1. Taxon sampling

In order to identify possible geographical patterns within *Isothecium alopecuroides*, a total of 66 samples were studied, of which 12 are from Morocco and exhibit the particular combination of characters mentioned above, 9 are from Southern Europe (Iberian Peninsula), 9 from Western Europe (United Kingdom, Ireland and France), 10 from Central Europe (Belgium, Switzerland and Germany), 6 from Eastern Europe (Georgia, Poland and Ukraine) and 20 from Northern Europe (Sweden and Norway). In addition, samples of the other European taxa mentioned above, as well as the Asian *I. subdiversiforme* Broth. and the N American *I. cristatum* (Hampe) H. Rob. were sampled. Attempts to use GenBank ITS accessions for two species appearing to be suitable as outgroups from the study of Ignatov and Huttunen (2002), *Hypnum cupressiforme* Hedw. and *Leucodon brachypus* Brid., were unsuccessful since these were impossible to align with those of the *Isothecium* species. Therefore, *Antitrichia curtipendula* (Hedw.) Brid. was used as outgroup. All samples included in the analysis are listed in Table 1.

2.2. Molecular methods

Total DNA was extracted using the DNeasy® Plant Mini Kit for DNA isolation from plant tissue (Qiagen). Double-stranded DNA templates were prepared by polymerase chain reaction (PCR). PCR was performed using Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech) in a 25 µl reaction volume according to the manufacturers' instructions.

For both molecular markers, the PCR programs given below were initiated by a melting step of 5 min at 95 °C and were followed by a final extension period of 8 min at 72 °C. For the internal transcribed spacer (ITS) the PCR program

Table 1
Locality information and GenBank Accession Nos. for specimens included in molecular analysis

Taxon		Collection locality, Collector, Herbarium	Genotype	GenBank ITS	GenBank tRNA-Gly
I. alopecuroides	T1	Ketama, J. Bou Bessoui, Morocco, *Cano* et al. *160397*, MUB	Iso2	DQ294869	DQ294771
	T2	Camaleño, Cantabria, Spain, *Guerra and Cano 080694*, MUB	Iso2	DQ294878	DQ294772
	T3	Calar del Mundo, Albacete, Spain, *Jiménez and Ros 170984*, MUB	Iso2	—	DQ294773
	T4	Bab Taza, J. Bouhalla, Morocco, *Albertos* et al. *150697*, S	Iso2	DQ294870	DQ294774
	T5	Ketama, J. Bou Bessoui, Morocco, *Cano* et al. *160397*, S	Iso2	DQ294871	DQ294775
	T6	Tetla Ketama, Morocco, *Draper 130502*, S	Iso2	DQ294872	DQ294776
	T7	Södermanland, Tyresö, Sweden, *Hedenäs 221191*, S	Iso2	DQ294898	DQ294777
	T8	Härjedalen, Sveg, Sweden, *Hedenäs 140789*, S	Iso2	DQ294899	DQ294778
	T9	Södermanland, Hölö, Sweden, *Hedenäs 120600*, S	Iso2	—	DQ294779
	T10	Västmanland, Sala, Sweden, *Hedenäs 070502*, S	Iso2	DQ294900	DQ294780
	T12	Caucasus Occidental, Chokhatauri District, Georgia, *Vasák 200780*, H	Iso2	DQ294916	DQ294782
	T14	Ketama, J. Bou Bessoui, Morocco, *Cano* et al. 150397, Bryology herbarium of the University Autónoma de Madrid	Iso2	—	DQ294784
	T15	Ketama, J. Bou Bessoui, Morocco, *Cano* et al. *160397*, Bryology herbarium of the University Autónoma de Madrid	Iso1	DQ294862	DQ294785
	T16	Ketama, Morocco, *Draper and Medina 110604*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294873	DQ294786
	T17	Ketama, Morocco, *Draper and Medina 110604*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294874	DQ294787
	T18	Tetla Ketama, Morocco, *Draper 130502*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294875	DQ294788
	T19	Ketama, J. Bou Bessoui, Morocco, *Cano* et al. *150397*, Bryology herbarium of the University Autónoma de Madrid	Iso1	DQ294863	DQ294789
	T20	Ketama, J. Bou Bessoui, Morocco, *Cano* et al. *160397*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294876	DQ294790
	T21	Ketama, J. Bou Bessoui, Morocco, *Cano* et al. *160397*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294877	DQ294791
	T22	Pto. de la Quesera, Segovia, Spain, *Lara 020990*, Bryology herbarium of the University Autónoma de Madrid	Iso2	—	DQ294792
	T23	Pto. de la Quesera, Segovia, Spain, *Albertos* et al. *110694*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294879	DQ294793
	T24	La Hiruela, Madrid, Spain, *Garilleti and Lara 011189*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294880	DQ294794
	T25	Lozoya del valle, Madrid, Spain, *Jorquera and Lara 091189*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294881	DQ294795
	T26	Lozoya del valle, Madrid, Spain, *Lara 020688*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294882	DQ294796
	T27	Malopolska Upland, Skole Gory, Poland, *Bednarek-Ochyra 300685*, S	Iso2	—	DQ294797
	T28	West Carpathians, Tatry Wysokie, Poland, *Wójcicki 090886*, S	Iso2	DQ294913	DQ294798
	T29	Western Beskid Mountains, Gorce Mountains, Poland, *Jedrzejko and Zarnowiec 081090*, S	Iso1	DQ294860	DQ294799
	T30	Western Beskid Mountains, Zywiec Beskid, Polica range, Poland, *Zarnowiec and Klama 121089*, S	Iso2	DQ294914	DQ294800
	T31	Ivano-Frankovsk Province, Kalush District, Ukraine, *Ignatov 140991*, S	Iso2	DQ294915	DQ294801
	T32	Pont Nedd Fechan, Glyneath Co., Wales, UK, *Hedenäs 080904*, S	Iso2	DQ294890	DQ294802
	T33	Kt. Solothurn, Weissenstein, Switzerland, *Hedenäs 040804*, S	Iso2	DQ294887	DQ294803
	T34	Corbion, Prov. Luxembourg, Belgium, *De Sloover 160585*, S	Iso1	DQ294856	DQ294804
	T35	Småland, Bankeryd, Sweden, *Hedenäs 240504*, S	Iso2	DQ294901	DQ294805
	T36	Västergötland, Habo, Sweden, *Hedenäs 240504*, S	Iso1	DQ294855	DQ294806
	T37	Dalsland, Amal, Sweden, *Hedenäs 160791*, S	Iso2	DQ294902	DQ294807
	T38	Värmland, Mangskog, Sweden, *Fransson 180788*, S	Iso2	DQ294903	DQ294808
	T39	Närke, Örebro, Sweden, *Hakelier 010495*, S	Iso2	DQ294907	DQ294809
	T40	Södermanland, Vardinge, Sweden, *Hedenäs 210504*, S	Iso2	DQ294904	DQ294810
	T41	Västergötland, Gustav Adolf, Sweden, *Hedenäs 260504*, S	Iso2	DQ294905	DQ294811
	T42	Dalarna, Norrbärke, Sweden, *Hedenäs 050686*, S	Iso2	DQ294906	DQ294812
	T43	Sogn and Fjordane, Höyanger, Norway, *Hedenäs 140704*, S	Iso2	DQ294892	DQ294813
	T44	Sogn and Fjordane, Höyanger, Norway, *Hedenäs 140704*, S	Iso2	DQ294893	DQ294814
	T45	Sogn and Fjordane, Höyanger, Norway, *Hedenäs 140704*, S	Iso2	DQ294894	DQ294815
	T46	Hordaland, Etne, Norway, *Hedenäs 170704*, S	Iso2	DQ294895	DQ294816
	T47	Hordaland, Kvinnherad, Norway, *Hedenäs 200704*, S	Iso2	DQ294896	DQ294817
	T48	Hordaland, Kvinnherad, Norway, *Hedenäs 200704*, S	Iso1	DQ294859	DQ294818
	T49	Hordaland, Osteröy, Norway, *Hedenäs 230704*, S	Iso1	DQ294853	DQ294819

Table 1 (*continued*)

Taxon		Collection locality, Collector, Herbarium	Genotype	GenBank ITS	GenBank tRNA-Gly
	T50	Hordaland, Vaksdal, Norway, *Hedenäs 240704*, S	Iso2	DQ294897	DQ294820
	T61	Kt. Solothurn, Weissenstein, Switzerland, *Hedenäs 040804*, S	Iso2	DQ294888	DQ294831
	T62	Hontanar, Toledo, Spain, *Pokorny* et al. *080303*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294883	DQ294832
	T63	Robledo del Mazo, Toledo, Spain, *Cortés and Pokorny230603*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294884	DQ294833
	T74	Baden-Württemberg, Katzenbachtal S Bad Niederman, Germany, *Nebel and Preußing 131104*, STU	Iso2	DQ294909	DQ294840
	T75	Baden-Württemberg, Neckargebiet, Germany, *Saner 141104*, STU	Iso2	—	DQ294841
	T77	Baden-Württemberg, Schwäbisch-Fränkischer Wald, Germany, *Sebald 060397*, STU	Iso2	—	DQ294842
	T79	Baden-Württemberg, Germany, *Koperski 300302*, STU	Iso2	DQ294910	—
	T81	Rheinl-Pf., Krs. Bitburg-Prüm, Germany, *Holz 240196*, STU	Iso2	DQ294911	—
	T82	Baden-Württemberg, Südschwarzwald, Germany, *Nebel and Schoepe 041095*, STU	Iso2	DQ294908	—
	T83	Baden-Württemberg, Neckarland, Schönbuch, Germany, *Preußing and Preußing 101102*, STU	Iso2	DQ294912	DQ294843
	T84	Vallee du Marcadau, Haut Pyrenees, France, *Tangney 051003*, NMW	Iso2	DQ294885	DQ294844
	T85	Cirque du Gavarnie, Haut Pyrenees, France, *Tangney 071003*, NMW	Iso2	DQ294891	DQ294845
	T86	Guzet Neige, Haut Pyrenees, France, *Tangney 081003*, NMW	Iso1	DQ294854	DQ294846
	T87	Vallee du Marcadau, Haut Pyrenees, France, *Tangney 061003*, NMW	Iso2	DQ294886	DQ294847
	T88	Fingle Brook, Cornwall, England, UK, *Tangney August01*, NMW	Iso1	DQ294858	DQ294848
	T89	Trevigue, Cornwall, England, UK, *Tangney 150400*, NMW	Iso1	DQ294857	DQ294849
	T90	Pont Nedd Fechan, Glyneath Co., Wales, UK, *Tangney 080904*, NMW	Iso1	DQ294861	DQ294850
	T91	Tollymore Forest Park, Down, Ireland, *Holyoak 020902*, NMW	Iso2	DQ294889	DQ294851
I. algarvicum	T52	Pico do Ferreiro, Madeira, Portugal, *Hedenäs 121091*, S	Iso2	DQ294867	DQ294822
	T53	P. Moniz, R. de Janela, Madeira, Portugal, *Hedenäs 080691*, S	Iso2	DQ294868	DQ294823
I. myosuroides	T51	Vega de Liébana, Cantabria, Spain, *Sérgio 060604*, S	Iso2	DQ294922	DQ294821
	T58	Sogn and Fjordane, Höyanger, Norway, *Hedenäs 140704*, S	Iso1	DQ294866	DQ294828
	T59	Taganana, Tenerife, Spain, *Lara 210993*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294924	DQ294829
	T60	Bab Taza, J. Bouhalla, Morocco, *Cano* et al. *170397*, Bryology herbarium of the University Autónoma de Madrid	Iso2	DQ294927	DQ294830
I. myosuroides var. brachythecioides	T66	West Galway, Ireland, *Long 130704*, S	Iso2	DQ294921	DQ294835
	T67	North Ebudes, Scotland, UK, *Long 290604*, S	Iso2	DQ294926	DQ294836
	T72	Hordaland, Tysnesoy, Norway, *Long 190791*, E	Iso2	DQ294925	DQ294838
I. stoloniferum	T55	Digby County, Nova Scotia, Canada, *Schofield 090590*, S	Iso2	—	DQ294825
	T56	E Winchelsea Island, mouth of Nanoose Bay, Vancouver, Canada, *Schofield 100697*, S	Iso2	DQ294920	DQ294826
I. holtii	T65	Rogaland, Sokndal, Norway, *Odland 210996*, BG	Iso2	DQ294923	DQ294834
	T68	Easter Ross, Scotland, UK, *Long 280788*, E	Iso1	DQ294865	DQ294837
	T73	Wicklow, Ballinaskea, Ireland, *Long 070488*, E	Iso1	DQ294864	DQ294839
I. cristatum	T54	Saturna Island, Fiddler's Cove, British Columbia, Canada, *Schofield 200597*, S	Iso2	DQ294919	DQ294824
I. subdiversiforme	T11	Sichuan Province, Omei Shan, China, *Redfearn* et al. *230888*, H	Iso2	DQ294917	DQ294781
	T13	Tottori Pref., Tottori, Mt. Kyuso, Japan, *Koponen 010981*, H	Iso2	—	DQ294783
	T57	Honshu, Gifu-ken, Japan, *Mizutani 261091*, S	Iso2	DQ294918	DQ294827
A. curtipendula	D30	Hälsingland, Kårböle, Mt. Kerstaberget, Sweden, *Hedenäs 240799*, S	Iso2	DQ294852	DQ294770

employed was 35 cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min 30 s at 72 °C, with the primers '18SF' and '26SR' (Rydin et al., 2004) or '18 F' and '25R' (Quandt et al., 2000). The number of these cycles was increased up to 50 for some samples with DNA difficult to amplify. The amplified fragment comprises the 3′ end of the small subunit ribosomal RNA gene (SSU rDNA), the internal transcribed spacer 1 (ITS1), the 5.8S ribosomal RNA gene (5.8S rDNA), the internal transcribed spacer 2 (ITS2), and the 5′ end of the large subunit ribosomal RNA gene (LSU rDNA). Ribosomal RNA genes' and spacers' borders have been determined by comparison with currently accepted secondary structure models of yeast (*Saccharomyces cerevisiae* Meyen ex Hansen) and *Arabidopsis thaliana* (L.) Heynh. (CRW database; Cannone et al., 2002) and verified by compensatory mutations where possible (terminal 5.8S rDNA vs. initial LSU). For tRNA-Gly, 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 1 min 30 s at 72 °C was employed, with the

primers 'trnGf' and 'trnGr' (Pacak and Szweykowska-Kulinska, 2000).

Amplified fragments were cleaned with a QIAquick™ Multiwell PCR Purification Kit (Qiagen) and eluted in 60–80 μl elution buffer (10 mM Tris–Cl, pH 8.5). Cycle sequencing was performed using the ABI BigDye Terminator Kit (Applied Biosystems) according to the instructions on the kit, and the sequencing products were cleaned using the DyeEx 96 Kit (Qiagen). The same primers as for the PCR were used. Sequencing products were resolved on an ABI 3100 automated sequencer. Double stranded sequencing was performed.

2.3. Sequence editing and alignment

Nucleotide sequence fragments were edited and assembled for each DNA region using the STADEN Package (http://www.mrc-lmb.cam.ac.uk/pubseq). The assembled sequences were first aligned using the ClustalW algorithm (Thompson et al., 1994), followed by manual adjustments. Regions of incomplete data in at the beginning and end of the sequences were identified and excluded from subsequent analysis, in total 32 5′ ITS1 and 3′ ITS2 positions were excluded from the first set of analysis and 36 tRNA-Gly positions from the second set of analysis. ITS1 and ITS2 regions that could not be unambiguously aligned due to prominent length polymorphisms were excluded from phylogenetic analysis as well as the SSU, 5.8S and LSU rDNA. The sequence alignments used in the analysis are available from I.D. on request. Strict consensus sequences were computed combining such samples of the same morphospecies that exhibited at the same time highly similar (less than 1% difference) rDNA and cpDNA sequences, and taking into account the phylogenetic position of these specimens in both phylograms (below).

While gaps in non-coding regions are often difficult to assess (Kelchner, 2000), parsimony analyses were run both with the insertions and deletions coded as informative characters (insertions/deletions coded as states; treatment INCL) and with the insertions and deletions themselves coded as uninformative (EXCL). Initial INCL and EXCL analyses were performed using the program NONA (Goloboff, 1993). The support values for well-supported branches was approximately the same irrespective of whether the gaps were included or not, and the trees resulting from the INCL and EXCL data sets of analysis differed mainly in portions with poorly supported clades (details not shown). Therefore, gaps were treated as missing data and generally length polymorphic regions (LPR) were excluded from the analyses.

2.4. Phylogenetic analysis

Parsimony and maximum likelihood (ML; via Bayesian analysis) were used as optimality criteria to compute phylogenetic trees; split decomposition (SD) and neighbour net (NN) analyses were used to compute distance based split networks. Clade support was assessed via non-parametric bootstrapping (Felsenstein, 1985; under parsimony), implemented in PAUP* 4.0 beta 10 (Swofford, 2002) and Bayesian inferred posterior probabilities (Huelsenbeck et al., 2001; Rannala and Yang, 1996).

Analysis under parsimony used a parsimony ratchet for PAUP* (PRAP) command block generated with PRAP 1.21 (Müller, 2004) using the default settings. Bootstrap values (BV) are based on 10,000 replicates. Bootstrap replicates used a simple addition tree as start, which was swapped by tree bisection reconnection, and one tree was held in memory. More exhaustive heuristic searches do not increase reliability of BV (Müller, 2005). Bayesian analysis was performed with MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and used one cold and three incrementally heated Monte Carlo Markov chains (MCMC) on two simultaneous runs. MCMC run for 1,000,000 generations, with trees sampled every 100th generation, each using a random tree as a starting point and a temperature parameter value of 0.2 (the default in MrBayes). The first 249 trees, in the case of ITS, and 892 trees, in the case of tRNA-Gly, of each run were discarded as burn-in, depending on when the MCMC log-likelihoods appeared to have become stationary and converged; the remaining trees were used to construct a Bayesian consensus tree and to infer posterior probabilities (PP). Models for Bayesian analysis were selected based on pair-wise likelihood ratio tests (LRT) and Akaike information criteria (AIC) of the 24 models implemented in MrModeltest 2.1 (Nylander, 2004). AIC and LRT preferred a general-time reversible (GTR) model plus a gamma shape parameter (Γ) for the ITS data (Rodríguez et al., 1990), and a HKY model (Hasegawa et al., 1985) plus a parameter modelling a proportion of invariable sites (I) for tRNA-Gly data.

Modules implement in SplitsTree 4.1 (Huson and Bryant, 2006) were used to compute split networks and consensus networks. We used uncorrected *p*-distances for split decomposition (Bandelt and Dress, 1992a,b; Huson, 1998) and neighbour net analysis (Bryant and Moulton, 2002, 2004) since no rate test is available for phylogenetic networks. Both methods characterize incompatibilities (incongruent splits) in the data in advance of any phylogenetic reconstruction and provide a collection of possible resolutions through reticulation. Consensus networks allow to visualize competing topologies among phylogenetic methods, bootstrap replicates, Bayesian partitions (confidence networks), and equally parsimonious trees.

3. Results

3.1. Sequence variation

The ITS data set (ITS1 and ITS2) comprises 126 variable sites (out of a total of 464; missing data, rRNA gene regions and LPR excluded); 76 were found to be parsimony informative. Sequence motifs of all excluded length polymorphic regions cannot be unambiguously aligned for the complete

ITS data, but are diagnostic for specific ITS variants (Fig. 1). Out of the 567 included tRNA-Gly sites, 28 out of 44 variable sites are parsimony informative. The amplified tRNA-Gly fragment exhibits three prominent indels (duplications), which have been excluded from analysis. The actual length variation of ITS1, ITS2 and tRNA-Gly is shown in Table 2.

Table 2
Actual length variation for the ITS1, ITS2 and tRNA-Gly sequences used in the study

	ITS1	ITS2	tRNA-Gly
I. algarvicum	256	261	532–543
I. alopecuroides s.l.	256–283	261–288	533–545
I. cristatum	256	262	525
I. holtii	257–267	263–270	526
I. myosuroides	257–267	270–288	526
I. myosuroides var. *brachytheciodes*	258	270	526
I. stoloniferum	258	262	526–533
I. subdiversiforme	251–267	271–272	526–554

3.2. Parsimony and ML analysis

3.2.1. General topology

Using *Antitrichia curtipendula* as outgroup, the basic topology of all most parsimonious trees (MPT) (15 in the case of ITS, 4 for tRNA-Gly) and the Bayesian consensus trees is identical for each data set; and the results of the ITS and tRNA-Gly analyses are largely congruent considering the terminal clades (Fig. 2); topological incongruence usually refers to the position of the distinguished clades to each other. Several specimens of *I. alopecuroides* (2 from Morocco and 9 from W Europe) form two well supported clades (clade A, BV = 100; PP = 1.0; and clade B, BV = 99.1; PP = 1.0) based on ITS data (Fig. 2A). Clade A comprises two Moroccan specimens of *I. alopecuroides* and is also well supported by tRNA-Gly data (BV = 99.9; PP = 1.0; Fig. 2B). Clade A is placed as a sister clade to all (ITS) or most (tRNA-Gly) of the remainder taxa of the ingroup. Specimens of clade B fall within the *Isothecium 2* clade based on tRNA-Gly data (Fig. 2B, below). Two specimens of *I. holtii* and one of *I. myosuroides* form a well-supported clade (C) based on ITS (BV = 99.5; PP = 1.0) and tRNA-Gly (BV = 100; PP = 0.99) data, which is sister to the *Isothecium 2* clade (ITS; Fig. 2A) or to all remaining specimens (tRNA-Gly; Fig. 2B). Specimens of *I. alopecuroides*, *I. algarvicum*, *I. cristatum*, *I. holtii*, *I. myosuroides* and *I. subdiversiforme* form a 'crown group' (*Isothecium 2* clade, Fig. 2). The *Isothecium 2* clade is supported by ITS data (BV = 74; PP = 1.0; Fig. 2A) and tRNA-Gly data (BV = 97; PP = 0.72; including the specimens of clade B; Fig. 2B).

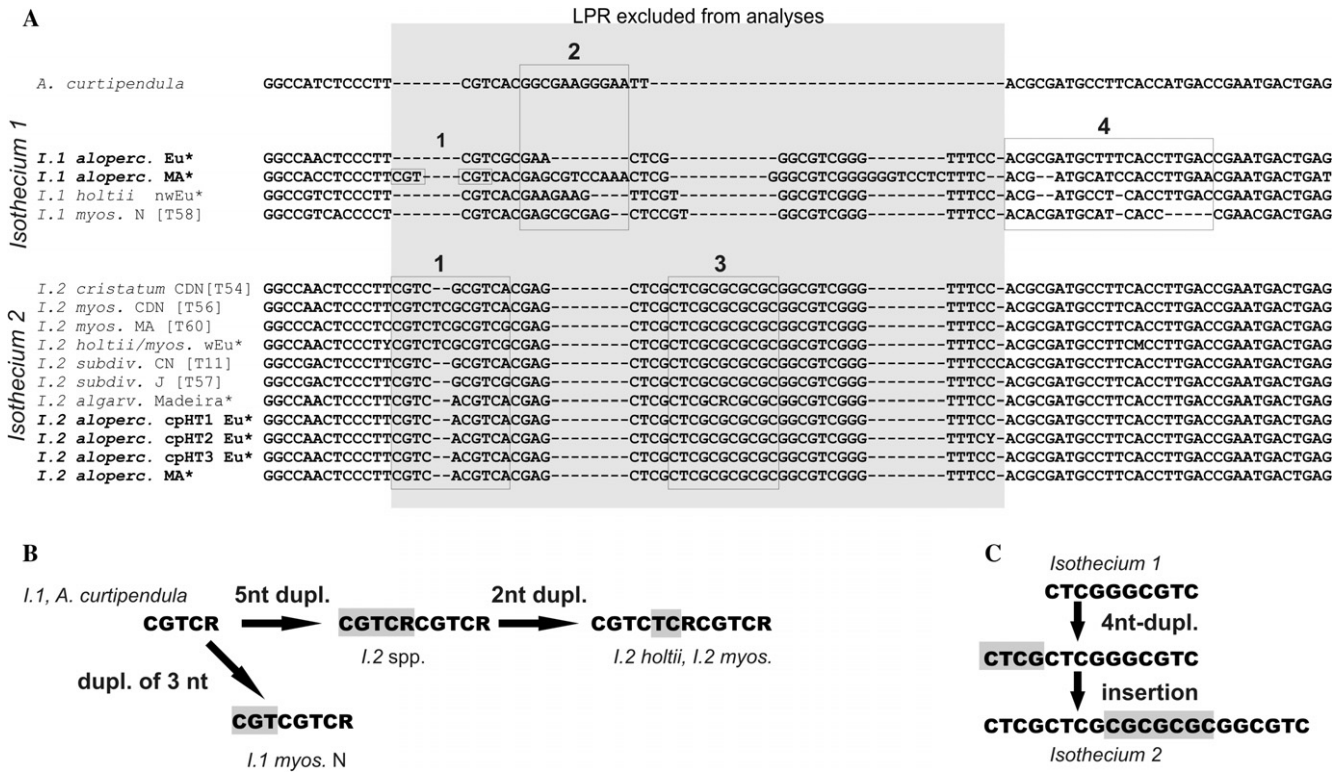

Fig. 1. Exemplary LPR of ITS data set. (A) Alignment of LPR and flanking ITS portions (alignment positions 96–196, ITS1); boxes stress highly diagnostic sequence features for *Isothecium 1* (boxes namely 1, 2, 4) and *Isothecium 2* (boxes namely 1, 3) individuals. Alignment of the LPR must be considered to be biased, whereas motif 4 can be aligned without artificial grouping of accessions. (B) Putative evolution of motif 1 characterized by duplication events. (C) Possible evolution of motif 3 diagnostic for *Isothecium 2*. *Isothecium1*/*Isothecium2* refer to two main clades showed in the phylogenetic trees (Fig. 2). cpHT1, cpHT2 and cpHT3 refer to the three chloroplast haplotypes identified in tRNA-Gly phylogenetic trees (Fig. 3b). I. myos. refers to *Isothecium myosuroides* s.l. Eu: Europe, MA: Morocco, N: Norway, CDN: Canada and CN: China. Near identical individuals are represented by strict consensus sequences (∗).

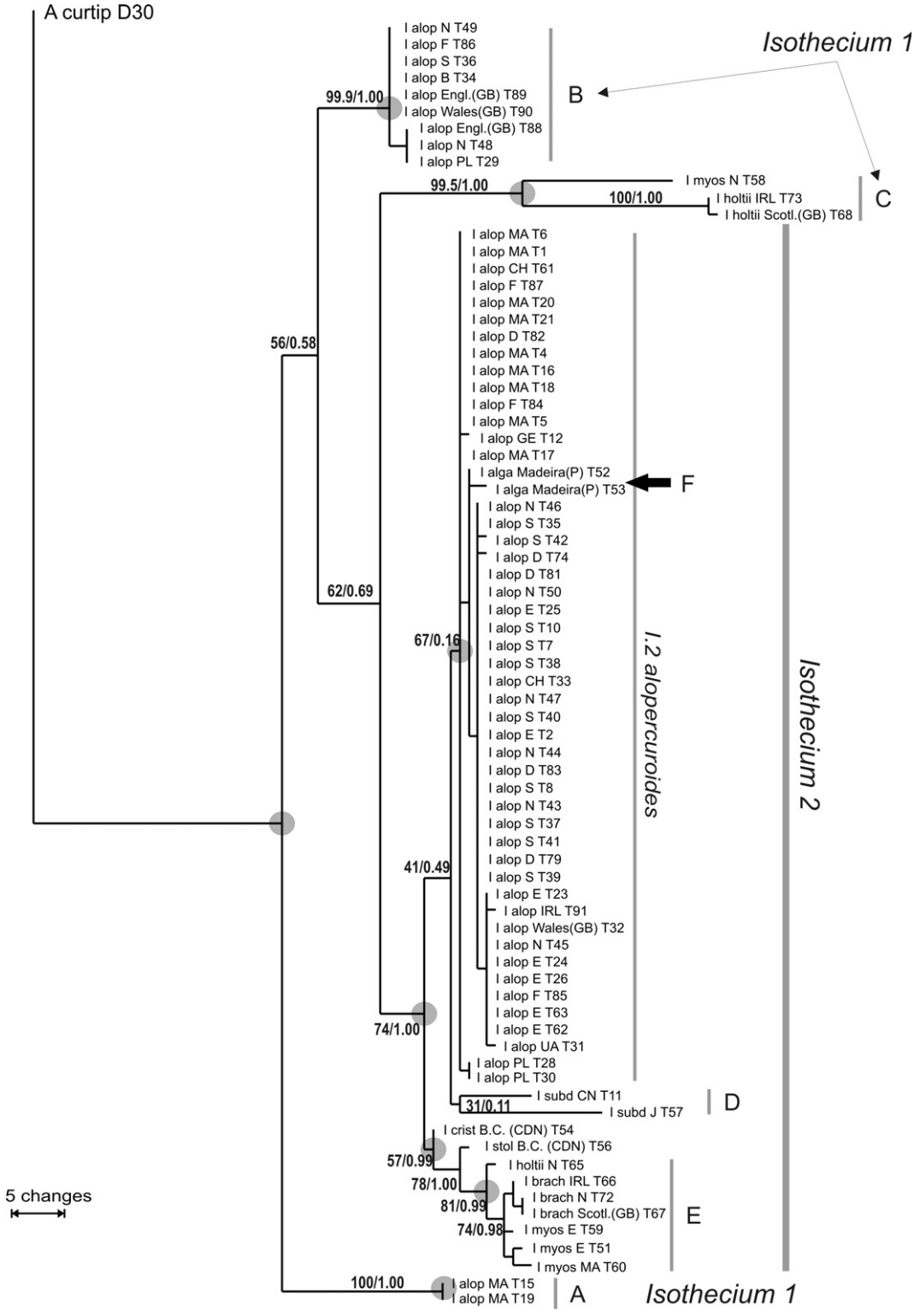

Fig. 2. Arbitrarily selected most parsimonious trees (MPT). Numbers on major branches indicate Bootstrap values (BV)/Posterior probabilities (PP). Abbreviations used for countries: B (Belgium), CDN (Canada: B.C.—British Columbia, N.S.—Nova Scotia), CH (Switzerland), CN (China), D (Germany), E (Spain), F (France), GB (Great Britain: Engl.—England, Scotl.—Scotland, Wales), GE (Georgia), IRL (Ireland), J (Japan), MA (Morocco), Mad (Madeira), N (Norway), PL (Poland), S (Sweden) and UA (Ukraine). *Isothecium 1*, *Isothecium 2* and clades referred to by large capital letters are discussed in the text. (A, this page) MPT ($L = 191$, CI = 0.801, RI = 0.916) for ITS data set. (B, next page) MPT ($L = 54$, CI = 0.9074, RI = 0.9597) for tRNA-Gly data set. Note that the large capital letter B refers to members of a clade identified by the ITS data (A).

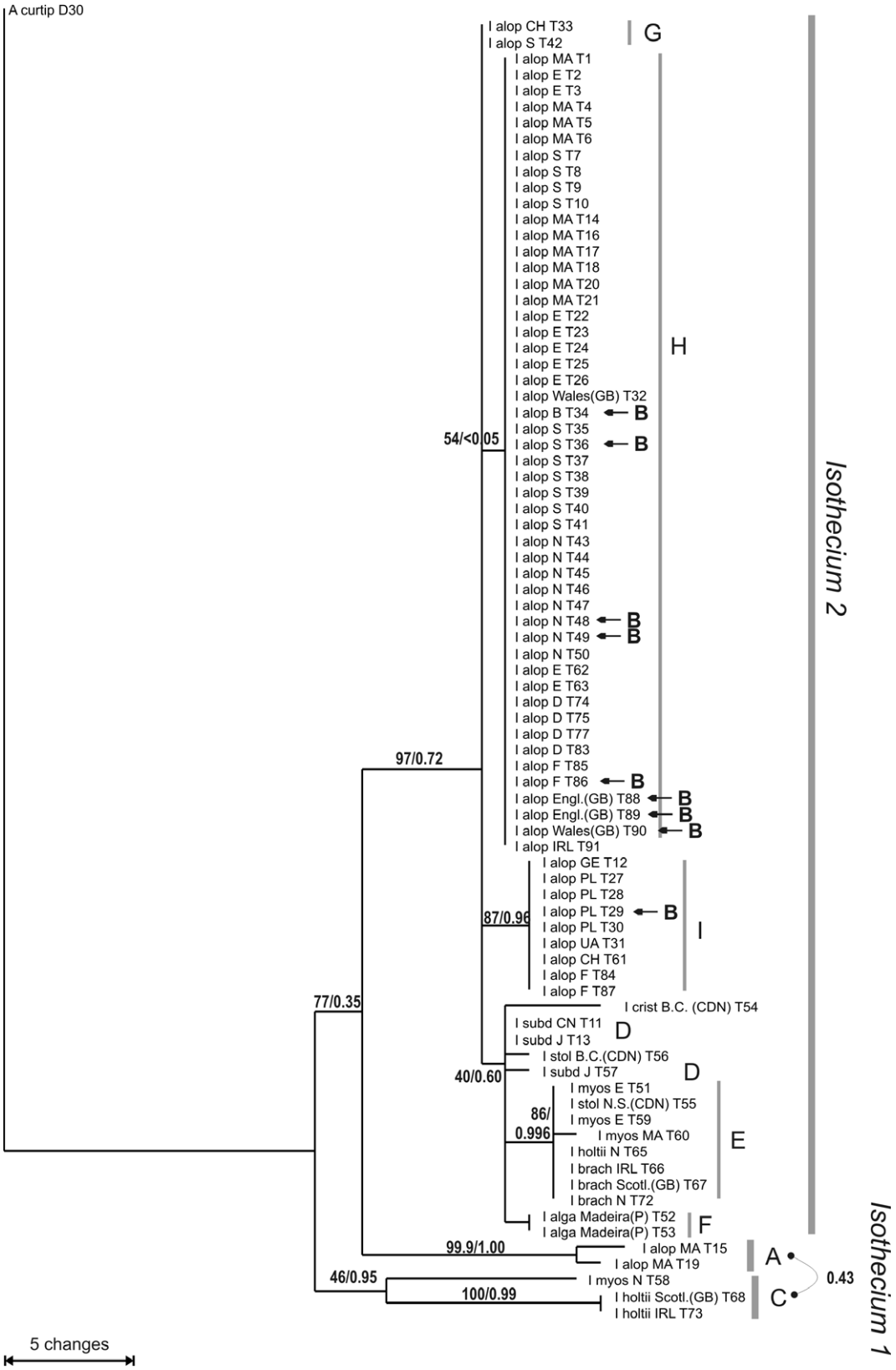

Fig. 2 (*continued*)

Within the *Isothecium 2* clade, specimens group basically according to their assigned morphotypes and several subclades (D–I) can be distinguished with low to high support based either on ITS data (e.g., clade D), tRNA-Gly data (clade I), or both data sets (clade E). The well supported (BV = 87; PP = 0.96) clade I (tRNA-Gly data; Fig. 2B)

includes specimens from mostly mountainous areas of France, Georgia, Poland, Switzerland and Ukraine. In the following, we will refer to the specimens of the clades A, B (ITS-based) and C as the *Isothecium 1* grade ("*I.1*") and to the specimens of the 'crown-group' (clades D–I) as the *Isothecium 2* clade ("*I.2*").

3.2.2. 'Crown group' differentiation

Differentiation within the 'crown-group' is generally higher within ITS1 and ITS2 data than within tRNA-Gly data, but most nodes within the 'crown-group' are only moderately to weakly supported by BV and PP. Highest support (BV = 81/86; PP = 0.99/0.996) is found for a clade E comprising specimens of *I.2 holtii* and *I.2 myosuroides* (all MPT, ITS/tRNA-Gly data sets, Fig. 2). *I.2 cristatum* is either recognized as sister taxon to the *I.2 holtii-myosuroides* clade (ITS data, BV = 57; PP = 0.99; Fig. 2A) or its position is unresolved (tRNA-Gly data; Fig. 2B). The positions of *I. subdiversiforme* from China and Japan (1 and 2 specimens, respectively, "clade D"), *I. algarvicum* (2 specimens, "clade F") and *I. alopecuroides* (48 specimens in ITS data set and 53 specimens in tRNA-Gly data set) are unresolved. Within the tRNA-Gly data set, three haplotypes (clades G, H and I; Fig. 2B) are found among *I.2. alopecuroides* specimens. Two of these haplotypes are also represented in the W European *I.1 alopecuroides* (clade B defined by ITS data). However, these clades do not correlate with ITS subclades. Further relationships between ITS subclades and cpDNA haplotypes remain obscure.

Low BV and PP can be due to data incompatibility and equally probable alternative phylogenetic splits. Competing alternative splits can be visualized by consensus networks, and can be evaluated using all split frequencies saved during bootstrapping and Bayesian analysis. As default, splits that occurred in >5% of bootstrap replicates and 'un-burned' Bayesian inferred ML phylograms are saved. The analysis of the consensus networks of all the MPT for the section containing the *Isothecium 2* crown-group (Fig. 3) showed alternative phylogenetic splits that especially affect to the positions of *I. subdiversiforme* (ITS data set) and *I. cristatum* (tRNA-Gly).

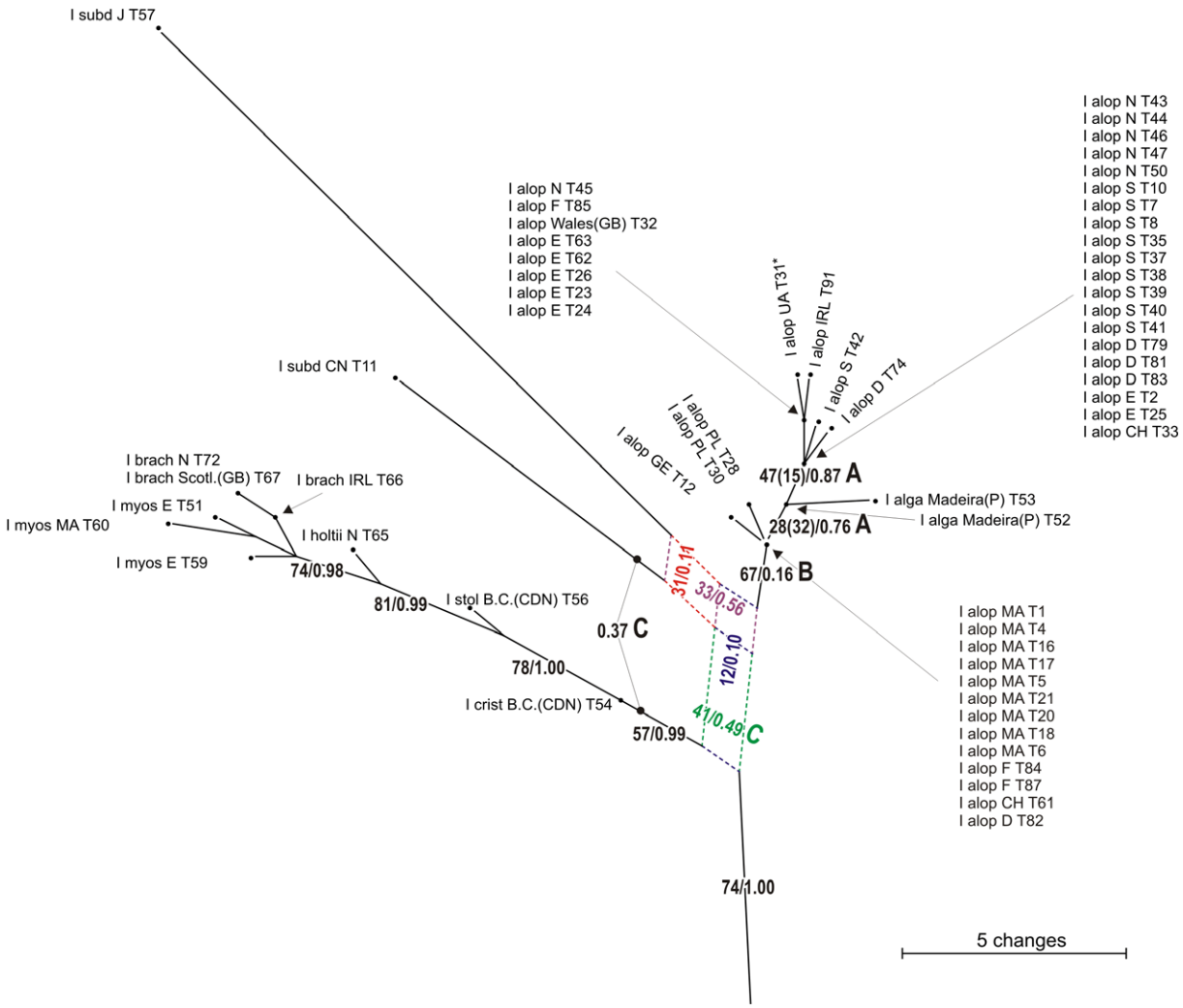

Fig. 3. Consensus network of all MPT. The section containing the *Isothecium 2* 'crown group' is shown. BV/PP values are given for the major branches. Abbreviations used for countries are the same as in Fig. 2. (A, this page) Consensus network for ITS data set. (B, next page) Consensus network for tRNA-Gly data set.

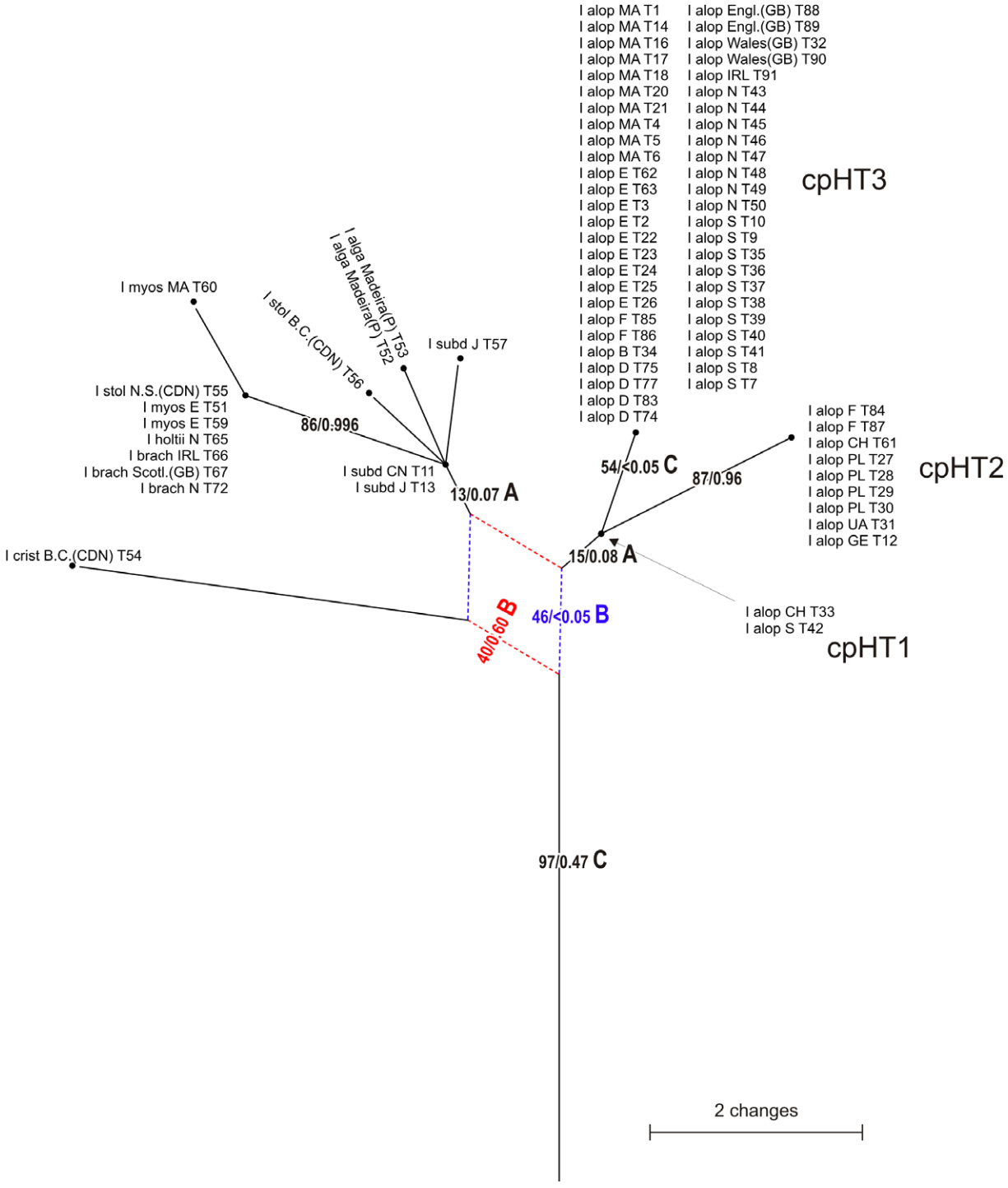

Fig. 3 (*continued*)

3.3. Analysis of incompatible split patterns

Strict consensus sequences were used instead of the original sequences to reduce the number of taxa and, hence, to further focus on relationships between such taxonomic units that are circumscribed by a unique combination of morphological characters and specific ITS and tRNA-Gly sequences. As expected in the case of highly divergent and complex structured data sets (Huson, 1998; Huson and Bryant, 2006), fewer splits are represented in the SD splits graph than in the NN splits graphs (Figs. 4 and 5). All splits detected in the SD analysis are also represented in the NN splits graphs, in particular the North American *I.2 cristatum–I.2 myosuroides* and the *I.2 subdiversiforme–Isothecium* 1 splits (fat edges in Fig. 5) are confirmed. Incongruent splits exhibited in the NN and SD splits graphs can be correlated to such nodes in parsimony and ML phylograms that lack highest support. Thus, low BV and PP support is not due to

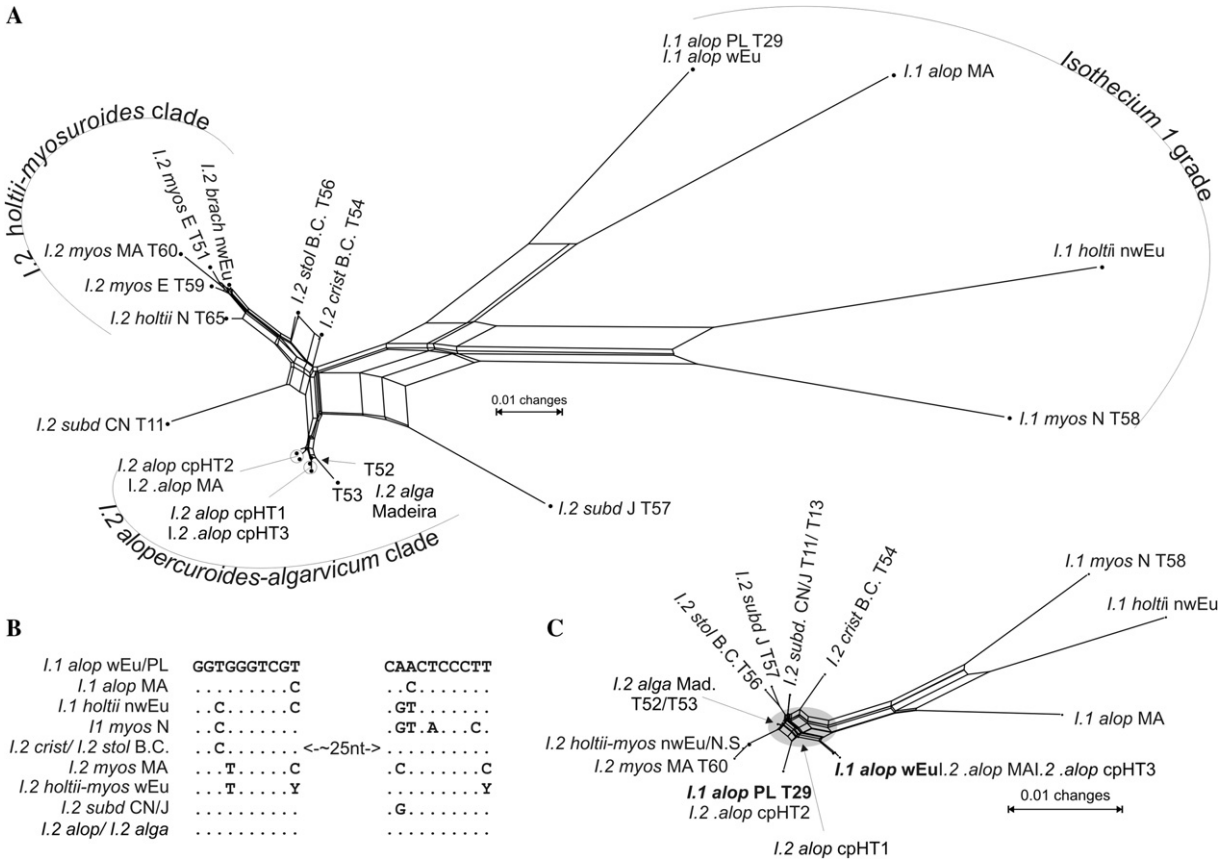

Fig. 4. Visualisation of incongruent splits among the used data by planar NN split networks. Individuals that share identical ($\leqslant$1 nt difference) ITS and tRNA-Gly sequences are represented by strict consensus sequences. (A) NN split network based on ITS data (uncorrected *p*-distances, LPR regions included, fit = 97.63). (B) Portion of the alignment (ITS1) exhibiting mainly nucleotides that produce splits incongruent to the phylogenetic tree hypotheses (see Figs. 2a and 3a). Labels refer to all individual accessions exhibiting the sequence shown. (C) NN split network based on the tRNA-Gly data (uncorrected *p*-distances, fit = 96.85). Bold font: *I.1 alopecuroides* exhibiting the identical tRNA-Gly sequences as their *I.2 alopecuroides* neighbours. Grey circle: members of the *Isothecium 2* clade.

the inability of the data to define nodes (lack of resolution) but due to incongruence within the used data sets. Partly overlapping split patterns indicate some evolutionary connection between *Isothecium 1* and several *Isothecium 2* specimens. For example, the predominant ITS-based split into *I.1* and *I.2* specimens and clades, respectively, competes with such splits that would indicate a closer relationship between the European *I.1 alopecuroides* and most crown-group (*I.2*) taxa (Fig. 4). This alternative ITS split correlates to tRNA-Gly split and the observation that all European *I.1 alopecuroides* exhibit cpDNA haplotypes 100% identical to *I.2 alopecuroides* cpDNA haplotypes. Analogous observations can be made for many incompatibilities between both data sets. For example, tRNA-Gly data place the specimens of *I.2 algarvicum* among a clade comprising all the *Isothecium 2* taxa except for *I.2 alopecuroides*, which is in contrast to the ITS analysis favouring a *I.2 algarvicum–alopecuroides* clade. In the NN analysis of ITS data, a split can be identified that relates the *I.2 alopecuroides–algarvicum* clades to *I.2 cristatum*, which is a sister group to the remaining *I.2* clades. General incongruence also affects the relationship among and between the *Isothecium 1* clades. Incongruent splits among the used data (indicated by box-like portions in Figs. 4 and 5) can be correlated with the alignment and affect predominantly the placement of the *I.2 subdiversiforme*, *I.2 cristatum* and *I.2 holtiilmyosuroides*, which share several point mutations with one or more *Isothecium 1* clades in contrast to the *I.2 alopecuroides*. ITS sequences of these individuals account for most of the phylogenetic incongruence that decreases support of according nodes in the phylogenetic trees (Figs. 2 and 3). In the case of tRNA-Gly data, dominant incongruent splits preclude the identification of sister relationships among the members of the *Isothecium 2* clade (grey circle in Fig. 4).

4. Discussion

4.1. Methodological appreciations: ITS "paralogy"

Phylogenetic inferences based on the nuclear and chloroplast markers do not recover long established morphological species as monophyletic units. Such results could potentially be due to sampling of different "paralogous" ITS copies (sensu Álvarez and Wendel, 2003; Bailey et al., 2003) in different individuals. "ITS paralogy" in that context (independent evolution of ITS variants obtained

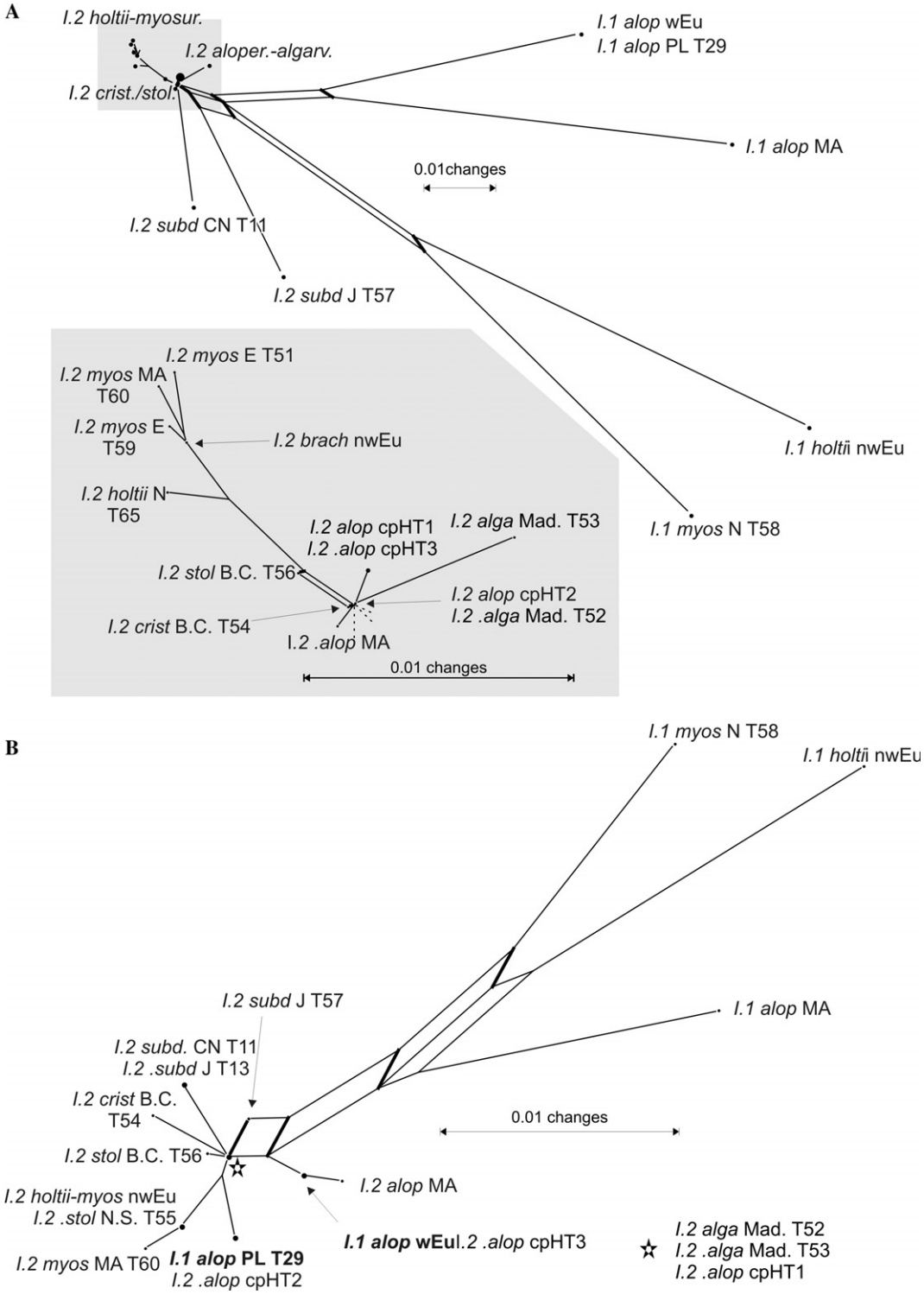

Fig. 5. Non-planar network reconstruction using SD method (uncorrected distances). (A) SD split network based on ITS data (fit = 61.16). The upper left portion of the graph is enlarged in the shaded box. (B) SD split network based on tRNA-Gly data (fit = 81.3).

from the same individual or distributed within a group of individuals) may arise from various phenomena: (1) Divergent ITS homoeologues are combined within one data set. Homoeologues can only be addressed in data sets containing polyploids (possibly also diploids). Therefore, this phenomenon could not be identified in haploid genomes such as the studied. (2) ITS "pseudogenes" (putatively non-functional ITS copies) are included in the data. In this study, the 5.8S rDNA is nearly identical in all *Isothecium* representatives, with a single fixed transition detected in *I.1 holtii*, *I.1 myosuroides*, Moroccan *I.1 alopecuroides* and the Chinese *I.2 subdiversiforme*, which rules out this phenomenon.

(3) Strict paralogy in a molecular genetic sense, i.e., gene paralogues forming a gene family. This phenomenon is not known in the case of the 35S rDNA cistron comprising the ITS1 and ITS2. (4) Incomplete lineage sorting (e.g., Comes and Abbott, 2001; Doyle, 1992; Neigel and Avise, 1986). In the present study, this phenomenon seems to have a minor effect since specific ITS variants strictly correlate to specific tRNA-Gly variants. With the exception of clade B within *I1. alopecuroides*, ITS data and tRNA-Gly data support the same taxonomic units. Even within the *Isothecium 2* clade, topological incongruence between both data sets can be attributed to the lack of resolution in either one of the data sets, e.g., all *I.2 alopecuroides* show one out of three possible tRNA-Gly haplotypes (clades G–I) and basically the same ITS variant. (5) Intraindividual ITS variability not linked to "paralogy", but to general heterogeneity among ITS copies as a result of incomplete concerted evolution and/or recombination, as it has been documented for higher plants using cloned ITS data sets (e.g., Denk et al., 2002, 2005; in the case of the beech tree). This phenomenon is normally considered to be rare in mosses (Shaw et al., 2002), and should be reflected by a number of polymorphic nucleotides in the directly sequenced PCR products.

In conclusion, "paralogy" effects in a broad sense do not have a recognizable effect on our analysis.

4.2. Morphological vs. molecular evidence

One major result of our molecular study was that each of the morphologically defined species (from here on called "morphospecies") *I. alopecuroides*, *I. holtii* and *I. myosuroides* is composed of 2–3 distantly related clades or phylogenetic species (potentially "cryptic species"). The second major result is that one of the potential cryptic species (of *I.2 alopecuroides*, *I.2 holtii* and *I.2 myosuroides*) falls within the *Isothecium 2* clade, which is defined by a group of closely related ITS and tRNA-Gly genotypes (note the lesser resolution and high similarity in generally divergent LPR of ITS1 and ITS2; Figs. 1–4). Accordingly, based on ITS or tRNA-Gly data, the genus *Isothecium* could be separated, respectively, into four or three clearly defined phylogenetic species or species aggregates (clades A–C, *Isothecium 2* clade), of which one, the *Isothecium 2* clade, includes the same morphospecies as each of the other three phylogenetic species. The later cannot be understood by simple cryptic speciation within *I. alopecuroides*, *I. holtii* and *I. myosuroides*.

One possible explanation for the lack of morphological correspondence with the molecular-based phylogeny could be that the morphospecies are currently defined by variable morphological characters. In this case, homoplasy is an artefact of inappropriate species circumscriptions. Although a relatively wide morphological variation has been observed within some morphospecies, such as *I. alopecuroides* and *I. myosuroides*, the diagnostic character states remain stable and no contradicting morphological patterns have so far been revealed (pers. obs. and treatment of these taxa in the European floras, e.g., Casas et al., 2001; Cortini Pedroti, 2006; Duell, 1992; Frey et al., 1995; Ignatov and Ignatova, 2003; Nebel and Philippi, 2001; Nyholm, 1965; Smith, 2004; Touw and Rubers, 1989). Before the present study, we performed morphometrical studies but no morphological pattern was found that suggested the revealed molecular groups. After the molecular results were at hand, we also carefully compared the morphology of three specimens pairs where one belongs to the ITS clade B and one to the large *I.2 alopecuroides* clade, and where the specimens in a pair were collected at closely situated localities: samples T29 and T30 from Poland, T36 and T41 from Sweden, and T47 and T48 from Norway. No morphological differences were found within any of the pairs, and neither were such patterns among the two clades evident. Similarly, the Moroccan *I.1 alopecuroides* individuals are morphologically indistinguishable from the Moroccan *I.2 alopecuroides* individuals.

A second possibility to explain the variation in morphology across the cladogram based on molecular data could be convergent morphological evolution. In *Isothecium* the most important gametophytic species-differentiating characters are found in the leaves, and one would thus expect that leaf characters should be easily modified by habitat factors if this is the correct explanation for the observed patterns. However, in a study of character state variation in pleurocarpous mosses across climatic zones, general habitat classes and the wetness gradient, relatively few leaf-related characters showed variation that was correlated with the habitat (Hedenäs, 2001). In addition, sporophyte orientation and the corresponding peristome characteristics are well correlated with gametophyte morphology among *Isothecium* species. Thus, if convergence would be the explanation for the found pattern, this must have affected not only the gametophyte but also, and at the same time, the much more complicated sporophyte. Since it appears very unlikely that convergence should have produced three distinct and well circumscribed morphospecies that in several cases consist of 2–3 molecularly distinct and relatively distantly related phylogenetic species, we discard convergence in morphology as the explanation for the observed patterns.

If the morphological characters are correctly identified and they are not homoplastic due to convergence, a third explanation for the lack of pattern in morphological characters with regard to the molecular based tree could be that the morphological characters are, at least to a large degree, not genetically fixed. In particular, the high morphological variation (as illustrated by the number of distinguished morphospecies) within the *Isothecium 2* clade is in some contrast to the low molecular differentiation within this clade. This could indeed reflect a general phenotypic plasticity within the genus, as it has been reported for other bryophyte taxa (e.g., Shaw and Allen, 2000; Shaw et al., 2003; Vanderpoorten et al., 2001; Vanderpoorten, 2004). Phenotypic plasticity could probably explain the lack of support for the morphospecies (e.g., *I. subdiversiforme*)

within this phylogenetic clade. Our morphotypes are largely independent of the ecological settings (above), hence, if morphological characters are generally plastic in *Isothecium*, one would expect that there is virtually no correlation between morphotypes and genotypes. However, ITS data allows distinguishing between *I.1 holtii* and *I.1 myosuroides* (clade C); as well as between *I.2 holtii* and *I.2 myosuroides*/*I.2 myosuroides* var. *brachythecioides* (clade E). Similarly, all the morphotaxa within the *Isothecium 2* clade can be traced by specific ITS and/or tRNA-Gly sequences, at least with low support. In the case of angiosperms, morphological differentiation has been often found to outrun ITS differentiation (e.g., Denk et al., 2002, 2005; Denk and Grimm, 2005). Divergence in cpDNA sequence data often is even lower, and even well accepted species may exhibit identical sequences (GenBank data, pers. obs.) Resultant data sets are comparable in the level of differentiation to our data for the *Isothecium 2* clade, and, as in our study, many nodes are poorly supported.

Finally, another alternative to interpret the lack of consensus between the morphological and molecular patterns could be the lateral transfer of genetic material among morphologically differentiated "species". This hypothesis is extensively discussed in the following section.

4.3. Morphospecies and their sequence properties

Our results indicate that morphology alone is inadequate to circumscribe species within *Isothecium.* More data are needed to resolve some relationships completely, but the most strongly supported parts of the cladograms and the NN and SD split networks suggest that the three European morphospecies *I. alopecuroides*, *I. holtii* and *I. myosuroides* are the result of a complex evolutionary history. Similarly surprising results were seen in other studies of mosses: in *Fontinalis* (Shaw and Allen, 2000), specimens of the morphospecies *F. antipyretica* Hedw. were found in two different clades, in one of these together with representatives of other, morphologically deviating species; in *Sphagnum* section *Subsecunda* (Shaw et al., 2005b) morphologically defined species did not form genetically coherent groups and intragenic recombination was detected among different groups of populations. Gene flow was also detected among both closely and more distantly related species within the section *Acutifolia* (Shaw et al., 2005a). In our study, the morphospecies of *Isothecium* are found in two main clades, namely *Isothecium 1* and *Isothecium 2*.

All our *Isothecium 2* representatives must have had a unique common origin, since all the LPR are highly similar within the *Isothecium 2* ITS sequences and can be readily distinguished from the *Isothecium 1* ITS variants and the outgroup *A. curtipendula.* This applies also for the tRNA-Gly data and indels. The LPR excluded from the phylogenetic analyses of all *Isothecium 1* clades are more similar to the outgroup in gross parameters such as LPR motif length and sequence than the LPR of *Isothecium 2* (Fig. 1). If *A. curtipendula* and the *Isothecium 1* representatives are excluded, the complete ITS1 and ITS2 can be unambiguously aligned and used for analysis. Vice versa, the unambiguously alignable portion of ITS1 and ITS2 cannot be sufficiently enlarged, if all the *Isothecium 2* specimens are excluded. If the condition in *Isothecium 2* is ancestral, this would imply that the *Isothecium 1* clades and *Antitrichia* are descendants of *Isothecium 2*, and that phenotypically identical morphotypes evolved independently and underwent very different rates of molecular evolution.

In both the ITS and tRNA-Gly data sets, generally higher genetic distances (NN, SD), expected changes (ML), or assumed point mutations (MP), characterize the *Isothecium 1* complex in contrast to the *Isothecium 2* complex. This is surprising considering the amount of morphological variation within both groups. It is now commonly accepted that fixation rates are variable at various levels. For example, in the case of ITS1 and ITS2 generally divergent and generally conserved domains can be addressed, which are common in all green plants (Hershkovitz and Lewis, 1996; Hershkovitz et al., 1999). Thus, the computed branch and edge lengths do not directly allow an *ad hoc* assumption about the evolutionary time span. However, more mutations have apparently been fixed in both regions during the evolution and differentiation of each *Isothecium 1* representative in comparison to their *Isothecium 2* morphologically identical counterparts. This can be best explained by two scenarios: either the *Isothecium 1* divergence occurred before the *Isothecium 2* complex differentiated into distinguishable haplotypes, or fixation rate is generally slowed down within the *Isothecium 2* complex, and, respectively, increased among the *Isothecium 1* taxa. The first scenario could indicate a recent differentiation and radiation of the *Isothecium 2* species, which would explain why European *I.1 alopecuroides* (stratigraphically older) exhibit *I.2 alopecuroides* cpDNA haplotypes (via introgression, if a more 'derived' *Isothecium 2* ITS variant and cpDNA haplotype intrograded into *I. holtii* and *I. myosuroides*, or via chloroplast capture). Under the second scenario, frequent interbreeding and/or hybridization among early *Isothecium* populations could have resulted in a homogenized gene pool, where many mutations could have accumulated primarily in the most isolated populations such as the *Isothecium 1* representatives, whereas the differentiation among *Isothecium 2* representatives was hindered. However, this scenario cannot fully explain the scarcity of *Isothecium 1* types in contrast to *Isothecium 2* types and the miscellaneous genetic structure of clade B. Hybridization events among *Isothecium* species were also suggested by Ryall et al. (2005) as a possible explanation for the observed patterns in a study focusing on the *I. myosuroides* complex. In that study, two specimens that were morphologically clearly of *I. stoloniferum* type did not belong to their molecularly well-supported *I. stoloniferum–I. myosuroides* clade. Ryall et al. (2005) also cited observations of meiotic irregularities in *Isothecium* as a possible support for the hybridization hypothesis (Ramsay and Schofield, 1981).

It seems clear that the current molecular data are not sufficient to reconstruct the phylogenetic relationships among the *Isothecium* "species", as originally defined by morphology. The four or five (if *I.1 myorsuroides* is treated as a distinct species) phylogenetic species based on the phylograms cannot define less ambiguous species: Three morphotypes would have evolved two or three times forming a cryptic species complex. However, one of the phylogenetic species (*Isothecium 2*) not only includes three of the "cryptic species" but is differentiated into subgroups. The subgroups of *Isothecium 2* show a general (although low) correlation between morphospecies and genotype. This ends up in the puzzling taxonomic situation that in the genus *Isothecium* "cryptic species" (where molecular differentiation outruns morphological differentiation) co-exist with a species aggregate (where morphological differentiation outruns molecular differentiation).

Possibly, this puzzle reflects reticulation or migration routes. It has to be noted that the molecular data was obtained from gametophytes, and haploid genomes are uniparentally inherited. The systematic loss of one parental lineage may interfere with any phylogenetic reconstruction, in particular, if reticulation is taken into account. Also, biogeographic signals may dominate over evolutionary signals. Considering the generation change in mosses, it could be possible that an originally monophyletic species lost large mainly non-coding parts of its original genome through introgression or other processes, but that portions responsible for its morphological characteristics were retained. Thus, *Isothecium holtii* or *I. alopecuroides* (originally all *Isothecium 1*) could survive with a high proportion of DNA intrograded from other species (*Isothecium 2*). Distinct rDNA and cpDNA variants could co-exist in the gametophytic populations (and be recognized as "cryptic species") and one parental lineage could be largely lost (explaining the numbers of *Isothecium 1* vs. *Isothecium 2* genotypes in *I. alopecuroides*, *I. holtii* and *I. myosuroides*). In the case of polyploid angiosperm hybrids, intragenomic competition between different parental rDNA arrays (rDNA homoeologues) has been studied and it has been demonstrated that one rDNA homoeologue can dominate another during expression in experimental F1 hybrids containing two sets of functional rDNA homoeologues (e.g., Komarova et al., 2004). In the case of the tetraploid *Nicotiana tabacum*, a stabilized hybrid, one parental rDNA was found to be completely eliminated (Lim et al., 2000; Volkov et al., 2004). These kinds of processes have not been so deeply studied in bryophytes as in tracheophytes. Nevertheless, interspecific hybridization has been proved to occur in several genera such as *Philonotis* (Buryová, 2004), *Plagiomnium* (Wyatt et al., 1992; Wyatt and Odrzykoski, 1998) and *Sphagnum* (e.g., Såstad et al., 2001). One of the most clear examples of these kind of reticulate evolution events was reported within the latter genus, were at least four taxa combine the nuclear sequences of one section with the chloroplast sequences of another (Shaw and Goffinet, 2000). This is similar to the situation of our clade B showing a *Isothecium 1* ITS type, but *Isothecium 2* tRNA-Gly type.

4.4. Tree building versus networks

As previously explained, the observed differentiation patterns within and between ITS and tRNA-Gly data sets cannot be accurately modelled by phylogenetic-tree building methods. Combining the data is difficult since the overall variability of tRNA-Gly data is significantly lower than the divergence detected in the ITS1 and ITS2. A combined analysis would require more sophisticated methods of inference such as ML using a mixed model (Bininda-Emonds et al., 2001; Moret et al., 2002; Stamatakis, pers. comm., 2006). Furthermore, the situation is complicated by the fact that some individuals exhibit ITS variants combined with cpDNA haplotypes that otherwise are diagnostic for more distant related individuals. Such a data structure requires reticulate evolutionary events, which cause a systematic error in phylogenetic tree-building methods. Non-parametric bootstrapping as well as Bayesian posterior probabilities have been designed to protect from sampling error but not from systematic error (Delsuc et al., 2005; Felsenstein, 1985; Huson and Bryant, 2006; Rannala and Yang, 1996). Considering the broad sampling of individuals and the highly divergent and partly incongruent differentiation patterns observed, systematic error is of higher importance in this case. Omitting the low supported nodes as unresolved does not significantly contribute to the understanding of this species complex and does not clarify the ambiguous results. By combining the classic phylogenetic tree building methods with detailed analysis of BV, PP and split networks, we are able to deal with the encountered phylogenetic incongruence (Grimm et al., 2006; Huson and Bryant, 2006) and to interpret the ambiguity within and between both data sets.

5. Conclusions

This study suggests molecular relationships that cut across since long established concepts of the morphologically defined species *Isothecium alopecuroides*, *I. holtii* and *I. myosuroides*. Convergent morphological evolution could potentially explain molecular variation within morphospecies, but is very unlikely in this particular case. Instead, the most strongly supported parts of the cladograms and the NN and SD split networks suggest that these species are the result of a complex evolutionary history, including exchange of genetic material (reticulation).

Additional molecular data sets have to be very carefully analysed and validated, respectively, constrained, by morphological, ecological and biogeographical evidence. In addition, future analysis of this group will profit from a broader sampling, in particular of East Asian and North American taxa, as well as with the inclusion of additional molecular markers (preferably a mtDNA marker) and

sporophyte-based data. This will be necessary to prove or falsify the lack of *Isothecium 1* haplotypes in non-European *Isothecium*, to investigate the frequency of the different haplotypes in the European populations, and to further investigate ancient and recent reticulate evolutionary events, such as chloroplast capture, hybridization and introgression. The situation may be complicated by the loss of particular molecular phylogenetic information in several lineages, and future analysis should aim at understanding and reconstructing the evolutionary pathways that lead to the recent morphologic and molecular puzzle, before any taxonomic revision (including the recognition of monophyletic and cryptic species) can be accomplished. Nevertheless, the already assembled data demonstrate that intrageneric molecular evolution in mosses is a fruitful and interesting field of investigation, in particular with respect to evaluate the possibilities and limitations of different phylogenetic methods, such as split networks, confidence networks and phylogenetic trees.

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References

Álvarez, I., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29, 417–434.

Bailey, C.D., Carr, T.G., Harris, S.A., Hughes, C.E., 2003. Characterization of angiosperm nrDNA polymorphism, paralogy, and pseudogenes. Mol. Phylogenet. Evol. 29, 435–455.

Bandelt, H.-J., Dress, A.W.M., 1992a. A canonical decomposition theory for metrics on a finite set. Adv. Math. 92, 622–628.

Bandelt, H.-J., Dress, A.W.M., 1992b. Split decomposition: a new and useful approach to phylogenetic analysis of distance data. Mol. Phylogenet. Evol. 1, 242–252.

Bininda-Emonds, O.R.P., Brady, S.G., Kim, J., Sanderson, M.J., 2001. Scaling of accuracy in extremely large phylogenetic trees. In: Proceedings of the 6th Pacific Symposium on Biocomputing PSB 2002, pp. 547–558.

Bryant, D., Moulton, V., 2002. NeighborNet: an agglomerative method for the construction of planar phylogenetic networks. In: Guigó, R., Gusfield, D. (Eds.), Algorithms in bioinformatics, Second International Workshop, WABI. Lecture Notes in Computer Science 2452, pp. 375–391.

Bryant, D., Moulton, V., 2004. Neighbor-Net: An agglomerative method for the construction of phylogenetic networks. Mol. Biol. Evol. 21 (2), 255–265.

Buryová, B., 2004. Genetic variation in two closely related species of *Phylonotis* based on isozymes. Bryologist 107 (3), 316–327.

Cannone, J.J., Subramanian, S., Schnare, M.N., Collett, J.R., D'Souza, L.M., Du, Y., Feng, B., Lin, N., Madabusi, L.V., Muller, K.M., Pande, N., Shang, Z., Yu, N., Gutell, R.R., 2002. The Comparative RNA Web (CRW) Site: An online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinformatics 3, 2.

Casas, C., Brugués, M., Cros, R.M., 2001. Flora dels briòfits's dels Països Catalans. I. Molses. 2ª Ed. Revisada. Institut d'Estudis Catalans, Barcelona.

Chiang, T.Y., Schaal, B.A., 1999. Phylogeography of North American populations of the moss species *Hylocomium splendens* based on the nucleotide sequence of internal transcribed spacer 2 of nuclear ribosomal DNA. Mol. Ecol. 8, 1037–1042.

Comes, H.P., Abbott, R.J., 2001. Molecular phylogeography, reticulation, and lineage sorting in Mediterranean *Senecio* sect. *Senecio* (Asteraceae). Evolution 55, 1943–1962.

Corley, M.F.V., Crundwell, A.C., Düll, R., Hill, M.O., Smith, A.J.E., 1981. Mosses of Europe and the Azores; an annotated list of species, with synonyms from the recent literature. J. Bryol. 11, 609–689.

Cortini Pedroti, C., 2006. Flora dei muschi d'Italia. Bryopsida (II parte). Antonio Delfino Editore, Roma.

Denk, T., Grimm, G.W., 2005. Phylogeny and biogeography of *Zelkova* (Ulmaceae sensu stricto) as inferred from leaf morphology, ITS sequence data and the fossil record. Bot. J. Linn. Soc. 147, 129–157.

Denk, T., Grimm, G., Stögerer, K., Langer, M., Hemleben, V., 2002. The evolutionary history of *Fagus* in western Eurasia: evidence from genes, morphology and the fossil record. Plant Syst. Evol. 232, 213–236.

Denk, T., Grimm, G.W., Hemleben, V., 2005. Patterns of molecular and morphological differentiation in *Fagus*: implications for phylogeny. Am. J. Bot. 92, 1006–1016.

Delsuc, F., Brinkmann, H., Philippe, H., 2005. Phylogenomics and the reconstruction of the tree of live. Nat. Rev. Genet. 6, 361–375.

Doyle, J.J., 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. Syst. Biol. 17, 144–163.

Draper, I., Hedenäs, L., 2005. The identity of *Isothecium marocanum* Thér. and Meyl. J. Bryol. 27 (2), 163.

Duell, R., 1992. Distribution of European and Macaronesian mosses. Additions and progress. Bryologische Beitrag 8/9 e, pp. 1–273.

Esser, C., Ahmadinejad, N., Wiegand, C., Rotte, C., Sebastiani, F., Gelius-Dietrich, G., Henze, K., Kretschmann, E., Richly, E., Leister, D., Bryant, D., Steele, M.A., Lockhart, P.J., Penny, D., Martin, W., 2004. A genome phylogeny for mitochondria among alpha-protobacteria and a predominantly eubacterial ancestry of yeast nuclear genes. Mol. Biol. Evol. 21, 1643–1660.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Frey, W., Frahm, J.-P., Fischer, E., Lobin, W., 1995. Die Moos—und Farnpflanzen Europas. Band IV. Gustav Fischer Verlag, Stuttgart.

Goloboff, P.A., 1993. Estimating character weights during tree search. Cladistics 9, 83–91.

Grimm, G.W., Renner, S.S., Stamatakis, A., Hemleben, V. 2006. A nuclear ribosomal DNA phylogeny of *Acer* inferred with maximum likelihood, splits graphs, and motif analysis of 606 sequences. Evol. Bioinformatics Online 2.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Hedenäs, L., 1992. Flora of the Madeiran Pleurocarpous Mosses (Isobryales, Hypnobryales, Hookeriales). J. Cramer, Berlin, Stuttgart.

Hedenäs, L., 2001. Environmental factors potentially affecting character states in pleurocarpous mosses. Bryologist 104, 72–91.

Hershkovitz, M.A., Lewis, L.A., 1996. Deep-level diagnostic value of the rDNA-ITS region. Mol. Biol. Evol. 13, 1276–1295.

Hershkovitz, M.A., Zimmer, E.A., Hahn, W.J., 1999. Ribosomal DNA sequences and angiosperm systematics. In: Hollingsworth, P.M., Bateman, R.M., Gornall, R.J. (Eds.), Molecular Systematics and Plant Evolution. Taylor and Francis, London, pp. 268–326.

Holland, B., Huber, K.T., Moulton, V., Lockhart, P.J., 2004. Using consensus networks to visualize contradictory evidence for species phylogeny. Mol. Biol. Evol. 21, 1459–1461.

Huelsenbeck, J.P., Ronquist, F., 2001. MrBayes: Bayesian inference of phylogeny. Bioinformatics 17, 754–755.

Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Evolution—Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294, 2310–2314.

Huson, D.H., 1998. SplitsTree: analyzing and visualizing evolutionary data. Bioinformatics 14 (1), 68–73.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23 (2), 254–267.

Huttunen, S., Ignatov, M.S., Müller, K., Quandt, D., 2004. Phylogeny and evolution of epiphytism in the three moss families Meteoriaceae, Brachytheciaceae and Lembophyllaceae. Monographs in Systematic Botany from the Missouri Botanical Garden 98, 328–361.

Ignatov, M.S., Huttunen, S., 2002. Brachytheciaceae (Bryophyta)—A family of sibling genera. Arctoa 11, 245–296.

Ignatov, M.S., Ignatova, E.A., 2003. Moss flora of the Middle European Russia vol. 2. Fontinalaceae-Amblystegiaceae. Moscow: KMK Scientific Press Ltd. pp. 609–944 (Arctoa vol. 11, suppl. 2).

Kelchner, S.A., 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. Ann. Mo. Bot. Gard. 87, 482–498.

Komarova, N.Y., Grabe, T., Huigen, D.J., Hemleben, V., Volkov, R.A., 2004. Organization, differential expression and methylation of rDNA in artificial *Solanum* allopolyploids. Plant Mol. Biol. 56, 439–463.

Lim, K.Y., Kovarik, A., Matyasek, R., Bezdek, M., Lichtenstein, C.P., Leitch, A.R., 2000. Gene conversion of ribosomal DNA in *Nicotiana tabacum* is associated with undermethylated, decondensed and probably active gene units. Chromosoma 109, 161–172.

Moret, B.M.E., Roshan, U., Warnow, T., 2002. Sequence-length requirements for phylogenetic methods. In: Guigó, R., Gusfield, D. (Eds.), Algorithms in bioinformatics, Second International Workshop, WABI. Lecture Notes in Computer Science, vol. 2452, pp. 343–356.

Müller, K., 2004. PRAP—calculation of Bremer support for large data sets. Mol. Phylogenet. Evol. 31, 780–782.

Müller, K., 2005. The efficiency of different search strategies for estimating parsimony, jackknife, bootstrap, and Bremer support. BMC Evol. Biol. 5, 58.

Nebel, M., Philippi, G. (Eds.), 2001. Die Moose Baden-Württembergs. Band 2. Ulmer, Stuttgart.

Neigel, J.E., Avise, J.C., 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation. In: Neigel, J.E., Avise, J.C. (Eds.), Evolutionary Processes and Theory. Academic Press, New York, pp. 515–534.

Nyholm, E., 1965. Illustrated Moss Flora of Fennoscandia. II Musci. Fasc. 5. Swedish Natural Science Research Council, Stockholm.

Nylander, J.A.A., 2004. MrModeltest. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Uppsala.

Pacak, A., Szweykowska-Kulinska, Z., 2000. Molecular data concerning alloploid character and the origin of chloroplast and mitochondrial genomes in the liverwort *Pellia borealis*. J. Plant Biotechnol. 2, 101–108.

Quandt, D., Tangney, R.S., Frahm, J.-P., Frey, W., 2000. A molecular contribution for understanding the Lembophyllaceae (Bryopsida) based on noncoding chloroplast regions (cpDNA) and ITS2 (nrDNA) sequence data. J. Hattori Bot. Lab. 89, 71–92.

Ramsay, H.P., Schofield, W.B., 1981. Observations on the cytology of mosses endemic to Western North America which occur in British Columbia. J. Hattori Bot. Lab. 49, 279–304.

Rannala, B., Yang, Z., 1996. Probability distribution of molecular evolutionary trees: A new method of phylogenetic inference. J. Mol. Evol. 43, 304–311.

Rodríguez, F., Oliver, J.L., Marín, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. 142, 485–501.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Ryall, K., Whitton, J., Schofield, W., Ellis, S., Shaw, A.J., 2005. Molecular phylogenetic study of interspecific variation in the moss *Isothecium* (Brachytheciaceae). Syst. Bot. 30, 242–247.

Rydin, C., Pedersen, K.R., Friis, E.M., 2004. On the evolutionary history of *Ephedra*: Cretaceous fossils and extant molecules. Proc. Natl. Acad. Sci. USA 101, 16571–16576.

Shaw, A.J., 2001. Biogeographic patterns and cryptic speciation in bryophytes. J. Biogeogr. 28, 253–261.

Shaw, A.J., Allen, B., 2000. Phylogenetic relationships, morphological incongruence, and geographic speciation in the Fontinalaceae (Bryophyta). Mol. Phylogenet. Evol. 16, 225–237.

Shaw, A.J., Goffinet, B., 2000. Molecular evidence of reticulate evolution in the peatmosses (*Sphagnum*), including *S. ehyalinum* sp. nov. Bryologist 103 (2), 357–374.

Shaw, A.J., McDaniel, S.F., Werner, O., Ros, R.M., 2002. New frontiers in bryology and lichenology, Phylogeography and phylodemography. Bryologist 105, 373–383.

Shaw, A.J., Cox, C.J., Goffinet, B., Buck, W.R., Boles, S.B., 2003. Phylogenetic evidence of a rapid radiation of pleurocarpous mosses (Bryophytina). Evolution 57 (10), 2226–2241.

Shaw, A.J., Cox, C.J., Boles, S.B., 2005a. Phylogeny, Species Delimitation, and Recombination in *Sphagnum* Section *Acutifolia*. Syst. Bot. 30, 16–33.

Shaw, A.J., Melosik, I., Cox, C.J., Boles, S.B., 2005b. Divergent and reticulate evolution in closely related species of *Sphagnum* section *Subsecunda*. Bryologist 108, 363–376.

Smith, A.J.E., 2004. The Moss Flora of Britain and Ireland, second Ed. Cambridge University Press, Cambridge.

Steele, M.A., 2005. Should phylogenetic models by trying to 'fit an elephant'? Trends Gen. 21, 307–309.

Swofford, D.L., 2002. PAUP*: Phylogenetic analysis using parsimony (* and other methods). Version 4. Computer program distributed by the National Illinois History Survey, Champaign, USA.

Såstad, S.M., Stenøien, H.K., Flatberg, K.I., Bakken, S., 2001. The narrow endemic *Sphagnum troendelagicum* is an allopolyploid derivative of the widespread *S. balticum* and *S. tenellum*. Syst. Bot. 26 (1), 66–74.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Touw, A., Rubers, W.V., 1989. De Nederlandse Bladmossen. Stichting Uitgeverij Koninklijke Nederlandse. Natuurhistorische Vereniging, Utrecht.

Vanderpoorten, A., 2004. A simple taxonomic treatment for a complicated evolutionary story: the genus *Hygroamblystegium* (Hypnales, Amblystegiaceae). Monogr. Syst. Bot. Missouri Bot. Garden 98, 320–327.

Vanderpoorten, A., Shaw, A.J., Goffinet, B., 2001. Testing controversial alignments in *Amblystegium* and related genera (Amblystegiaceae: Bryopsida). Evidence from rDNA ITS sequences. Syst. Bot. 26 (3), 470–479.

Volkov, R.A., Medina, F.J., Zentgraf, U., Hemleben, V., 2004. Molecular cell biology: organization and molecular evolution of rDNA, nucleolar dominance and nucleolus structure. In: Esser, K., Lüttge, U., Beyschlag, W., Murata, J. (Eds.), Progress in Botany. Springer-Verlag, Berlin, Heidelberg, New York, pp. 106–146.

Werner, O., Ros, R.M., Cano, M.J., Guerra, J., 2002. *Tortula* and some related genera (Pottiaceae, Musci): phylogenetic relationships based on chloroplast rps4 sequences. Plant Syst. Evol. 235, 197–207.

Wyatt, R., Odrzykoski, I.J., 1998. On the origins of the allopolyploid moss *Plagiomnium cuspidatum*. Bryologist 101 (2), 263–271.

Wyatt, R., Odrzykoski, I.J., Stoneburner, A., 1992. Isozyme evidence of reticulate evolution in mosses: *Plagiomnium medium* is an allopolyploid of *P. ellipticum* × *P. insigne*. Syst. Bot. 17 (4), 532–550.

Winkworth, R.C., Bryant, D., Lockhart, P.J., Havell, D., Moulton, V., 2005. Biogeographic interpretation of splits graphs: least squares optimization of branch lengths. Syst. Biol. 54 (1), 56–65.