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Narrow species concepts in the *Frullania dilatata–appalachiana–eboracensis* complex (Porellales, Jungermanniopsida): evidence from nuclear and chloroplast DNA markers

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Abstract We investigated the phylogeny of a Holarctic-Asian group of *Frullania* species, the *Frullania dilatata–F. appalachiana–F. eboracensis* complex, using multiple accessions of morphologically circumscribed taxa and three molecular markers (nrITS region, cp DNA *trn*L-F and *atp*B-*rbc*L regions). Maximum parsimony and likelihood analyses indicated monophyly of morphologically defined taxa. Our phylogenies support a species rather than a subspecies concept within the complex, with four species in North America (*F. appalachiana*, *F. eboracensis*, *F. parvistipula* and *F. virginica*), and two species in Europe (*F. dilatata* and *F. parvistipula*). Accessions of *F. dilatata* from Southeast Europe and Asia are separated from other European accessions, indicating a former disjunct range of the species.

Keywords Chloroplast DNA · Distribution range · *Frullania* · Holarctic · nrITS · Porellales

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Introduction

The cosmopolitan liverwort genus *Frullania* Raddi is easily recognized by its reddish colour and the incubously, narrowly inserted, complicate-bilobed leaves with the lobule often forming a water-sac. Many representatives of this genus form large mats, and are important elements of the cryptogamic flora of tropical forests. *Frullania* is also common in temperate regions of the world. Due to its commonness and large size, *Frullania* has attracted many researchers who have set up more than 1,000 binomials that may represent an estimated number of 300–375 valid morphospecies (Schuster 1992; Gradstein et al. 2001).

Only recently has *Frullania* been studied using DNA sequence evidence (Hentschel et al. 2009; Heinrichs et al. 2010). These studies uncovered unexpected relationships and pointed to the need for a refined subgeneric classification. In this context, it was shown that the common Holarctic-Asian *F. tamarisci* (L.) Dumort. is a complex of at least eight biological species with rather narrow distribution ranges (Heinrichs et al. 2010).

In the study reported here, we investigated the phylogeny of another common Holarctic-Asian group of *Frullania species*, the *F. dilatata* (L.) Dumort.–*F. appalachiana* R. M. Schust.–*F. eboracensis* Gottsche complex (Schuster 1992). Representatives of this complex are widely distributed throughout the Holarctic, and show a "mosaic distribution of taxonomic criteria" (Schuster 1992: p. 157). This diffuse distribution of character states has led to different taxonomic hypotheses and conclusions about the relationships among taxa (Hattori 1978; Rüegsegger 1986; Schuster 1992; Grolle and Long 2000). Members of the complex share short, helmet-shaped lobules and rounded lobes lacking ocelli, and they grow closely adnate to their substrate. The species differ in their perianth surface (entire

to tuberculate), length of perianth beak, frequency and type of vegetative distribution, development of cell wall thickenings, and underleaf shape.

Materials and methods

Taxon sampling and outgroup selection

The taxa studied are listed in Table 1, including GenBank accession numbers and voucher details. DNA vouchers were revised or identified using the determination keys and descriptions provided by Paton (1999), Rüegsegger (1986), Schuster (1992) and Sim-Sim (1999). All taxa were represented by multiple accessions.

Ingroup taxa represent the *F. dilatata–appalachiana–eboracensis* complex and its sister species *F. azorica* Sim-Sim et al., and were selected according to the global *Frullania* phylogeny of Hentschel et al. (2009) and the treatments of Hattori (1978) and Schuster (1992). Based on these papers, *F. appalachiana*, *F. dilatata*, *F. eboracensis*, *F. parvistipula* Steph. [*F. eboracensis* subsp. *parvistipula* (Steph.) R.M.Schust. and *F. virginica* Gottsche [*F. eboracensis* subsp. *virginica* (Gottsche) R.M.Schust.] were regarded as members of the complex. The morphologically similar *F. britoniae* A. Evans (Schuster 1992) is not closely related to the *F. dilatata–appalachiana–eboracensis* complex (Hentschel et al. 2009) and was therefore not included in this study. *F. plana* Sull. was designated as outgroup taxon for phylogenetic reconstruction.

DNA extraction, PCR amplification and sequencing

Plant tissue from the distal portions of a few shoots was isolated from collections housed in the herbaria GOET, JE, SAAR, UBC or UNA. Total genomic DNA was purified using an Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) prior to amplification. Protocols for PCR were carried out as described previously: *trn*L-F region as described by Gradstein et al. (2006), *atp*B-*rbc*L intergenic spacer as described by Feldberg et al. (2010), and nrITS1-5.8S-ITS-2 region as described by Hartmann et al. (2006). Bidirectional sequences were generated using a MegaBACE 1000 automated sequencing machine using DYEnamic ET Primer DNA sequencing reagent (Amersham Biosciences, Little Chalfont, UK). Sequencing primers were those used for PCR (104 sequences were newly generated for this study; 14 sequences were downloaded from GenBank).

Phylogenetic analyses

All sequences were aligned manually in Bioedit version 7.0.5.2 (Hall 1999). Ambiguous positions were excluded from the alignment and lacking parts of sequences were coded as missing. Maximum parsimony (MP) and maximum likelihood (ML) analyses were carried out with PAUP* version 4.0b10 (Swofford 2000).

MP heuristic searches were conducted with the following options: heuristic search mode, 100 random-addition-sequence replicates, tree bisection-reconnection (TBR) branch swapping, MULTrees option on, and collapse zero-length branches off. All characters were treated as equally weighted and unordered. Nonparametric bootstrapping values (Felsenstein 1985) were generated as heuristic searches with 1,000 replicates, each with ten random-addition replicates. The number of rearrangements was restricted to ten million per replicate. Bootstrap percentage values (BP) above 70 were regarded as good support (Hillis and Bull 1993). Where more than one most parsimonious tree was found, trees were summarized in a strict consensus tree.

The three genomic regions were first analysed separately to check for incongruence. The strict consensus trees of the nonparametric bootstrap analyses were compared by eye to identify conflicting nodes supported by at least 70% (Mason-Gamer and Kellog 1996). The trees showed no evidence of incongruence. Hence, the datasets were combined.

Modeltest version 3.7 (Posada and Crandall 1998) in conjunction with PAUP* was used to select a model of evolution for the ML analyses of the combined dataset, performing a hierarchical likelihood ratio test and the Akaike information criterion. For the ML analyses a general time reversible model (Tavaré 1986) was chosen with among-site rate heterogeneity modelled as a discrete gamma distribution with four rate categories, and its estimated parameters (Γ) (Goldman 1993). The analysis was performed as a heuristic search using five random-sequence addition replicates, MULTrees option on, collapse zero length branches off, and TBR branch swapping. The confidence of branching was assessed with PAUP* using 200 nonparametric bootstrap resamplings generated as heuristic searches. The number of rearrangements was restricted to 5,000 per replicate.

Results

Of a total of 2,248 molecular characters, 264 were parsimony informative, 50 autapomorphic, and 1,934 constant (Table 2). The MP analysis resulted in 78 trees of 362 steps with a consistency index of 0.93 and a retention index of 0.98. The strict consensus tree is depicted in Fig. 1. A single most likely tree (ln = −5306.4422, Fig. 2) was found in the ML analysis. The ML topology largely resembled the MP topology.

Table 1 Taxa used in the present study, including information about the origin of the studied material, voucher information, and the herbarium where the voucher is deposited, as well as GenBank accession numbers

Taxon	Origin	Voucher (Herbarium[a])	GenBank Acc. Nos.		
			nrITS	*trn*L-F	*atp*B-*rbc*L
F. appalachiana R. M. Schust.	USA., North Carolina (I), Haywood Co., 1,500 m	Davison 7888 (UNA)	HQ330382	HQ330418	HQ330449
F. appalachiana	USA., North Carolina (II), Jackson Co., 1,650 m	Davison & Smith 6389 (UNA)	HQ330383	HQ330419	HQ330450
F. appalachiana	USA., North Carolina (III), Jackson Co., 1,600 m	Davison & Smith 6221 (UNA)	HQ330384	HQ330420	HQ330451
F. azorica Sim-Sim, Sérgio, Mues & Kraut	Madeira (I), Seixal, 380 m	Schäfer-Verwimp & Verwimp 25607 (GOET)	HQ330385	HQ330421	HQ330452
F. azorica	Madeira (II), Ribeiro do Tristão, 200 m	Eckstein 487 (GOET)	HQ330386	HQ330422	HQ330453
F. azorica	Madeira (III)	Mues 3743 (SAAR)	HQ330387	HQ330423	HQ330454
F. azorica	Madeira (IV)	Mues 3748 (SAAR)	HQ330388	HQ330424	HQ330455
F. dilatata (L.) Dumort.	British Isles, Cornwall, Tregony, 80 m	Wilson & Bolton Bryo 02255 (GOET)	HQ330389	–	HQ330456
F. dilatata	Bulgaria, Rhodope Mts., Bačkovo	Hentschel Bryo 0758 (GOET)	HQ330375/ **FJ380434**	**FJ380270**	HQ330457
F. dilatata	Georgia (I), Lesser Caucasus, Bordshomi, 1,300–1,700 m	Zündorf 21940 (JE)	HQ330390	HQ330425	HQ330458
F. dilatata	Georgia (II), Greater Caucasus, Shatili, 1,700 m	Zündorf 23914 (JE)	HQ330391	–	–
F. dilatata	Greece, Euböa, Ag. Mammas, 980 m	Bergmeier s.n. (GOET)	HQ330392	HQ330426	HQ330459
F. dilatata	Germany (I), Baden-Wurttembergia, Herdwangen-Schönach, 580 m	Schäfer-Verwimp & Verwimp 28687 (GOET)	HQ330393	HQ330427	HQ330460
F. dilatata	Germany (II), Baden-Wurttembergia, Öhningen, 595 m	Schäfer-Verwimp & Verwimp 28697 (GOET)	HQ330394	HQ330428	HQ330461
F. dilatata	Germany (III), Hesse, Bad Sooden-Allendorf, 240 m	Hentschel & Heinrichs Bryo 03137 (GOET)	HQ330395	HQ330429	HQ330462
F. dilatata	Germany (IV), Saarland, Enzweiler, 330 m	Sesterhenn 5474 (SAAR)	HQ330396	HQ330430	HQ330463
F. dilatata	Germany (V), Rhineland-Palatinate, Alzey-Worms, 140 m	Oesau DNA3 (GOET)	HQ330397	HQ330431	HQ330464
F. dilatata	Germany (VI), Baden-Wurttembergia, Wangen-Allgäu, 580 m	Schäfer-Verwimp & Verwimp 28826 (GOET)	HQ330398	HQ330432	HQ330465
F. dilatata	Germany (VII), Baden-Wurttembergia, Zell im Wiesental, 726 m	Schäfer-Verwimp & Verwimp 28670 (GOET)	HQ330399	HQ330433	HQ330466
F. dilatata	Germany (VIII), Hesse, Wendershausen, 191 m	Eckstein 6896 (GOET)	HQ330400	HQ330434	HQ330467
F. dilatata	Italy (I), Apulia, Pugnochiuso	Sauer 3090 (GOET)	HQ330401	HQ330435	HQ330468
F. dilatata	Italy (II), Trentino, Folgarida, 1,280 m	Schäfer-Verwimp & Verwimp 28466 (GOET)	HQ330402	HQ330436	HQ330469
F. dilatata	Italy (III), Trentino, Folgarida, 950 m	Schäfer-Verwimp & Verwimp 28452 (GOET)	HQ330403	HQ330437	HQ330470
F. dilatata	Norway, Aust-Agder, Kristiansand	Hentschel Bryo 03221 (JE)	HQ330404	–	HQ330471
F. dilatata	Switzerland, Niwalden, Wolfenschiessen, 910 m	Hentschel & Busch Bryo 02764 (JE)	HQ330405	HQ330438	HQ330472
F. dilatata	Turkey, Antalya, Bey Daglari, 150 m	Schäfer-Verwimp & Verwimp 20703/T (GOET)	HQ330406	HQ330439	HQ330473
F. eboracensis Gottsche	Canada, Nova Scotia (I), Victoria Co., Warren Lake	Schofield et al. 96568 (UBC)	HQ330407	HQ330440	–
F. eboracensis	Canada, Nova Scotia (II), Queens Co., Jake's Landing area	Schofield & Belland 96881 (UBC)	HQ330408	HQ330441	–
F. eboracensis	Canada, Prince Edward Island, Dalvay Woodlands	Belland 16039 (UBC)	HQ330409	HQ330442	–
F. eboracensis	USA., Alabama, Jackson Co., 500 m	Davison 6875 (UNA)	HQ330410	HQ330443	HQ330474
F. eboracensis	USA., North Carolina (I), Haywood Co., 1,500 m	Davison 7889 (UNA)	HQ330411	HQ330444	HQ330475
F. eboracensis	USA., North Carolina (II), Swain Co., 600 m	Davison & Smith 7424 (GOET)	HQ330412	HQ330445	HQ330476
F. eboracensis	USA., North Carolina (III), Swain Co., 600 m	Davison & Smith 7390 (UNA)	HQ330413	–	–
F. eboracensis	USA., New York, Albany Co., Clarksville, 180 m	Smith 50725 (UBC)	HQ330414	HQ330446	–
F. eboracensis	USA., West Virginia, Pocahontas Co., 600–900 m	Davison 5193 (GOET)	HQ330376/ **FJ380437**	**FJ380273**	–
F. parvistipula Steph.	Georgia, Lesser Caucasus, Bordshomi, 1,900 m	Zündorf 21852 (JE)	HQ330415	–	HQ330477
F. parvistipula	Italy, Sicily, SE Trapani, 760 m	Eckstein 4684 (GOET)	HQ330377/ **FJ380438**	**FJ380274**	–
F. parvistipula	USA., New Mexico (I)	Worthington 32814 (GOET)	HQ330378/ **FJ380433**	**FJ380269**	–
F. parvistipula	USA., New Mexico (II), Otero Co., Sacramento Mts., 2,600 m	Worthington 31081 (GOET)	HQ330379/ **FJ380435**	**FJ380271**	–
F. plana Sull.	USA., West Virginia, Pocahontas Co., 600–900 m	Davison 5236 (GOET)	HQ330380/ **FJ380432**	**FJ380268**	HQ330478

Table 1 continued

Taxon	Origin	Voucher (Herbarium[a])	GenBank Acc. Nos.		
			nrITS	*trn*L-F	*atp*B-*rbc*L
F. plana	USA., Alabama, Marshall Co., 200 m	Davison 4325 (GOET)	HQ330381/ **FJ380431**	**FJ380267**	HQ330479
F. virginica Gottsche	USA., Louisiana, Rapides Parish	Hyatt s.n. (GOET)	HQ330416	HQ330447	HQ330480
F. virginica	USA., Alabama, Winston Co., 180 m	Davison 3550 (GOET)	HQ330417	HQ330448	–

Sequences in bold were obtained from GenBank

[a] Herbarium acronyms follow Holmgren et al. (1990)

Table 2 Distribution of constant and phylogenetically informative sites for aligned positions of the three genomic regions

	*atp*B-*rbc*L	*trn*L-F	ITS1-5.8S-ITS2	Total
Number of sites in matrix	704	451	1,093	2,248
Constant	663	423	848	1,934
Autapomorphic	11	4	35	50
Parsimony informative	30	24	210	264

The robust *F. azorica* clade is placed sister to a well-supported clade representing the *F. dilatata–appalachiana–eboracensis* complex. This clade is divided into two clades with BP values of >91. One clade includes multiple accessions of the Eurasian *F. dilatata*, the other clade well bootstrap supported lineages with multiple accessions of *F. parvistipula* (Georgia, Italy, North America), *F. appalachiana* (North America), *F. eboracensis* (North America) and *F. virginica* Gottsche (North America). *F. virginica* is sister to a clade with *F. appalachiana* and *F. eboracensis* in a well-supported sister relationship. *F. parvistipula* forms the sister to the remainder of the complex.

Discussion

Species versus subspecies concept in the *F. dilatata–appalachiana–eboracensis* complex

Species concepts in bryophytes are still largely based on morphology. Taxonomic assessments of morphological character states are often subjective, leading to author-dependent taxonomic conclusions and fluctuations in estimates of species numbers (Heinrichs et al. 2003). Several taxa of the *F. dilatata–appalachiana–eboracensis* complex have alternatively been treated as species (Grolle 1970; Hattori 1978; Rüegsegger 1986) or subspecies (Schuster 1992). Schuster's argument for a classification of *F. virginica* and *F. parvistipula* as subspecies of *F. eboracensis* was the "mosaic distribution of taxonomic criteria" rather than a lack of discrete characters that would allow a morphological identification of related accessions. On the other

hand, Schuster (1983) established species rank for *F. appalachiana*, another taxon closely related to the above complex. He regarded this taxon as most closely related to *F. dilatata* (Schuster 1983, 1992).

Molecular data allow an evaluation of morphology-based hypotheses (Heinrichs et al. 2009b; Huttunen and Ignatov 2010). In our study, *F. appalachiana* is placed sister to *F. eboracensis* s.str. with high bootstrap support, and not closely related to *F. dilatata*. The position of *F. appalachiana* within the *F. eboracensis–F. virginica–F. parvistipula* clade requires species rank for all taxa, or alternatively, the reduction of *F. appalachiana* to a subspecies of *F. eboracensis*. We favour species rank because the taxa occur partially sympatrically and can usually easily be identified using morphological evidence. Species rank is also supported by the molecular trees with high bootstrap percentage values for all investigated taxa (Figs. 1 and 2). According to our study, the complex is represented in North America by four species (*F. appalachiana*, *F. eboracensis*, *F. parvistipula*, *F. virginica*), whereas two species occur in Europe (*F. dilatata*, *F. parvistipula*).

Biogeography

With the exception of *F. parvistipula*, all ingroup representatives have more or less contiguous ranges (Fig. 1). The rather narrow distributional ranges of most ingroup species resemble the situation in the *F. tamarisci* complex (Heinrichs et al. 2010) with several species endemic to parts of the North American continent. The narrow distributional ranges of *F. eboracensis*, *F. virginica* and *F. appalachiana* are perhaps surprising in light of their high dispersal ability. Schuster (1992) explicitly points out that the Eastern North American *F. eboracensis* is an aggressive colonizer that copiously produces sporophytes. Despite the frequent presence of sporophytes and vegetative reproductive structures (caducous leaves), this species has not colonized Western North America, or other regions of the world. It remains to be tested if the diaspores of this species have a limited germination capacity after long-distance transportation (Van Zanten and Pócs 1981).
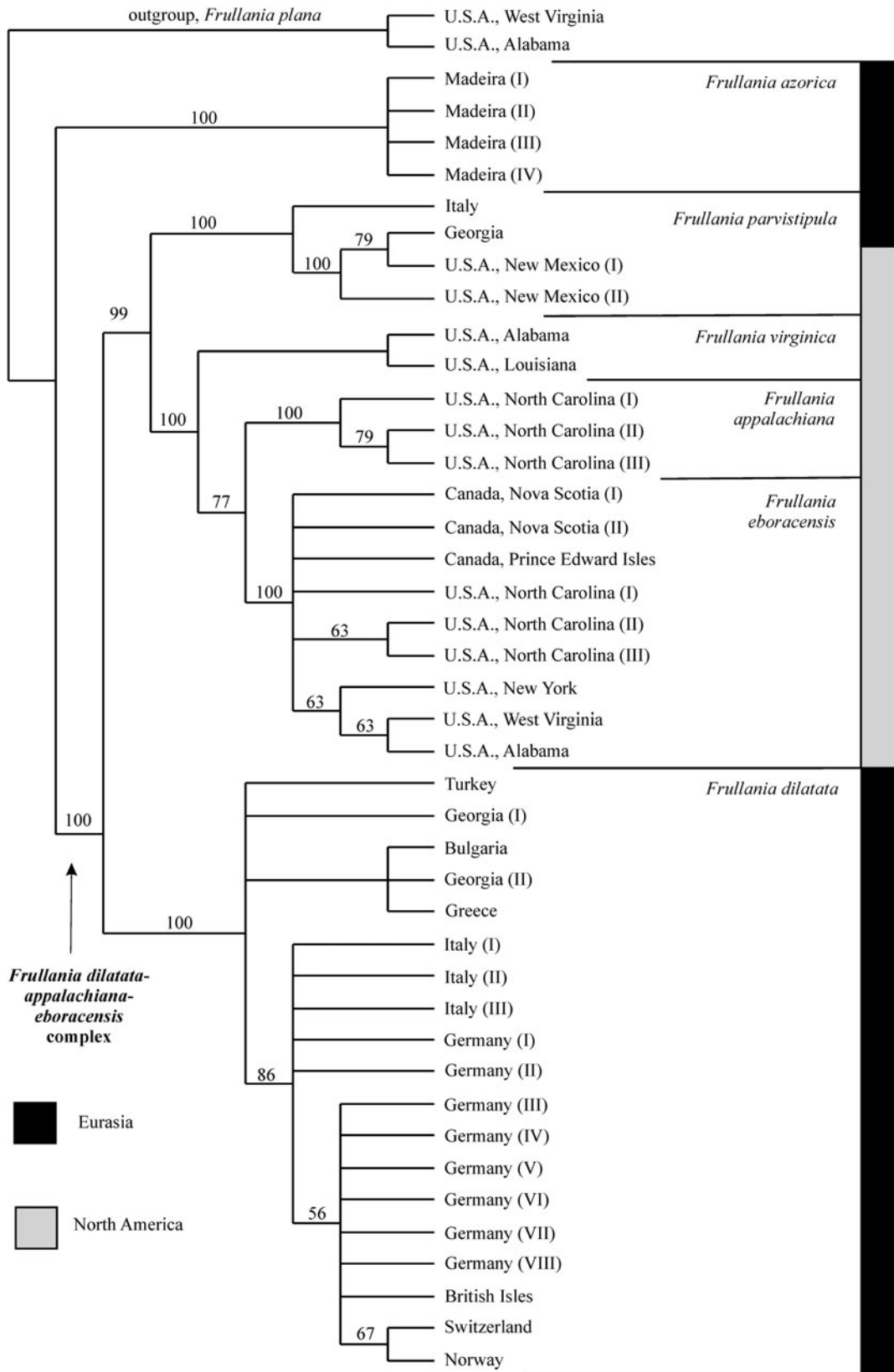
Fig. 1 Strict consensus of 78 equally parsimonious trees with bootstrap percentage values of >50 indicated on the branches. Eurasian or North American origin of the accessions is indicated by *shaded boxes*
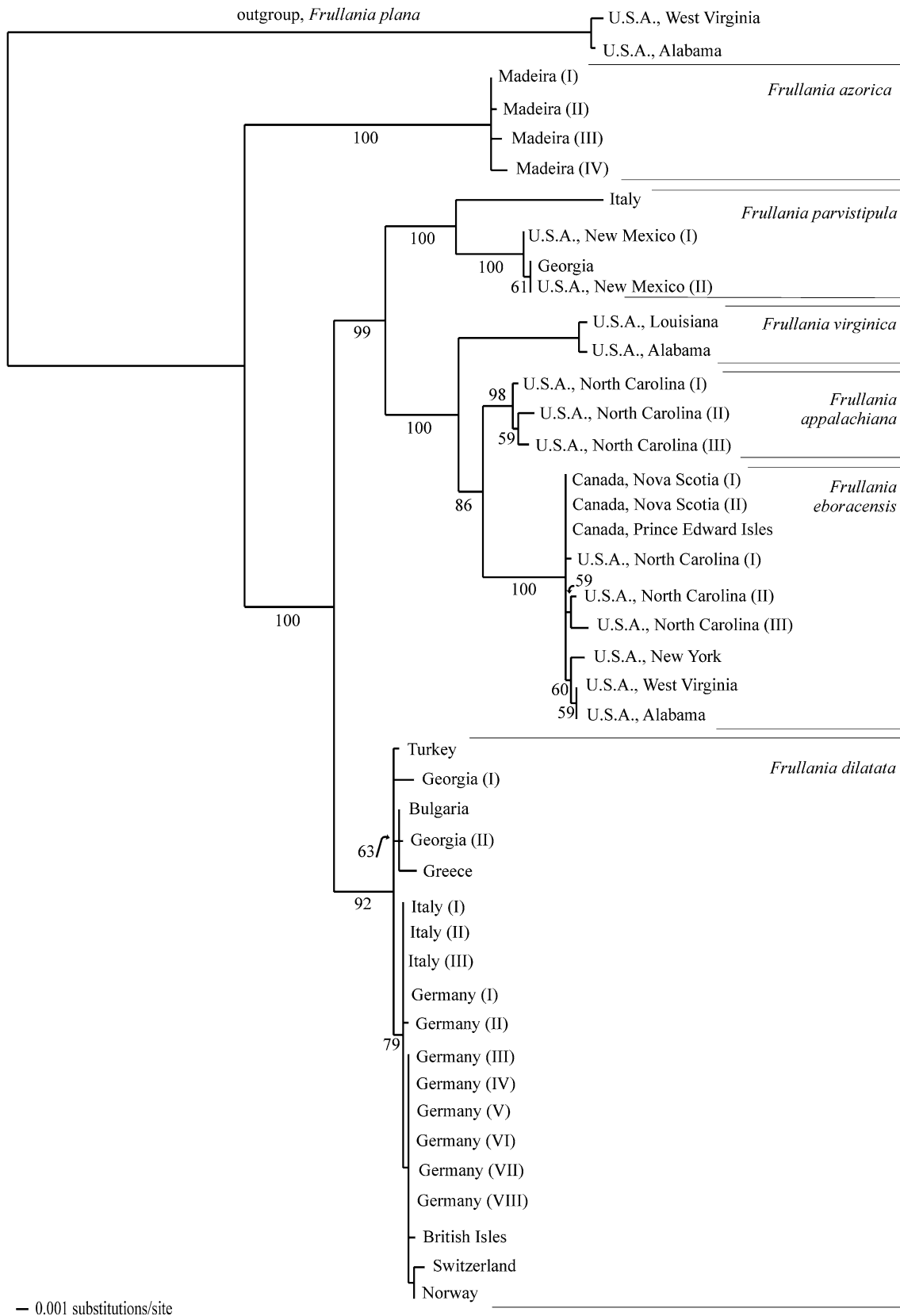
Fig. 2 The single most likely phylogram (ln = −5306.4422) resulting from the ML analysis of the combined molecular dataset. Bootstrap percentage values of >50 are indicated on the branches

Sequence variation within most ingroup representatives is low, providing some evidence for a relatively recent emergence of these species or extinction and recolonization processes as a consequence of the climate fluctuations of the Pleistocene (Hewitt 2000; Stewart and Lister 2001). Despite the limited sequence variation, there is internal structure within *F. dilatata* that is related to geography. Accessions from Southeast Europe and Asia are separated from other European accessions with good bootstrap support, pointing to a former disjunct range of this species.

Holarctic *Frullania* species seem to contradict the trend towards a general acceptance of intercontinental ranges of bryophyte species; however, our study confirms the monophyly of the North American–Eurasian range of *F. parvistipula*. Our study corroborates the view that *Frullania* is a genus that includes both narrow endemics such as *F. appalachiana* and intercontinentally distributed species such as *F. ericoides* (Nees) Mont. (Hentschel et al. 2009). Intercontinental ranges of bryophytes have been confirmed in several recent molecular studies (Shaw et al. 2003; Davison et al. 2006; Heinrichs et al. 2009b; Yu et al. 2010); however, their frequency seems to vary from genus to genus.

Future prospects

Molecular data partly confirm previous morphology-based hypotheses; however, there is rapidly growing evidence that morphology alone does not allow an understanding of actual bryophyte diversity (Shaw et al. 2008; Fuselier et al. 2009; Hedenäs 2009). Existing data point to underestimation of the *Frullania* species diversity by reason of conservative morphology within species complexes (Heinrichs et al. 2010). Hence, appraisals of species numbers and distributional ranges should be based on molecular plus morphological evidence (Heinrichs et al. 2009a). We urgently need more species-level phylogenies with extensive population sampling to approximate the actual diversity of *Frullania*, and to elucidate speciation processes and distribution range formation.

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