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How many species of *Isothecium* (Lembophyllaceae, Bryophyta) are there in Macaronesia? A survey using integrative taxonomy

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Species boundaries are sometimes difficult to assess, especially when molecular data do not neatly match morphologically defined units. This study investigates the moss genus *Isothecium*, with special emphasis on Macaronesian populations. Morphological studies are combined with the analysis of three rapidly evolving markers: nuclear internal transcribed spacer and plastid *trnG* and *trnL-trnF*. The results of the morphological studies suggest that *Isothecium* is represented by five species in Macaronesia, including a new endemic species from Madeira, ***Isothecium montanum* sp. nov.**, which is described here. The molecular results are less conclusive than the morphology results in delimiting species of this genus, even when indels are included as informative. Once possible methodological shortcomings have been discarded, the results can be interpreted as having been caused by incomplete lineage sorting, probably as a consequence of recent speciation. The molecular results also suggest that the origin of the Macaronesian endemics may be explained by at least two independent colonization events. Finally, the delimitation of a new endemic species of *Isothecium* in Macaronesia indicates that current knowledge on the taxonomy of spore-producing plants may be far from complete in this hotspot of biodiversity. *Botanical Journal of the Linnean Society*, 2015, **177**, 418–438.

ADDITIONAL KEYWORDS: *Isothecium algarvicum* – *Isothecium montanum* – *Isothecium prolixum* – taxonomy – species delimitation.

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INTRODUCTION

Macaronesia, including the Azores, Madeira, the Canary Islands and Cape Verde (but see Vanderpoorten, Rumsey & Carine, 2007), is one of the best-known phytogeographical regions, partly due to its high levels of diversification and adaptive radiation (e.g. Kim *et al*., 2008; see also Carine *et al*., 2004, and references therein). However, the species diversity varies substantially among archipelagos and plant groups. For instance, the number of radiations and single-island endemics in flowering plants is considerably lower in the Azores than in the other archipelagos (Carine & Schaefer, 2010). Recent molecular analyses revealed that the number of overlooked Azorean genetic entities is comparatively much higher than the number of taxa based on floristic data alone (Díaz-Pérez *et al*., 2008; see Schaefer *et al*., 2011, for a review). Regarding plant groups, the Macaronesian endemic bryophyte flora is clearly poorer than the endemic angiosperm flora. For instance, angiosperm single-island endemics represent 70% (567 species) of the endemic flora of the Canary Islands (Arechavaleta *et al*., 2009), but there is only one such bryophyte species (Patiño *et al*., 2013b). Moreover, only a few moss genera include more than one endemic species in Macaronesia (Vanderpoorten *et al*., 2010; see Patiño *et al*., 2014, for a review).

Analysis of the levels of endemism in the Macaronesian bryoflora is, however, under revision, especially since the use of molecular data has become more widely used in taxonomy and has helped to re-evaluate the status of numerous Macaronesian bryophyte endemics in recent years. For instance, some endemics have been synonymized with more broadly distributed taxa on the basis of their genetic similarity, such as *Fissidens luisieri* P. de la Varde (Werner *et al*., 2009), *Plagiochila allorgei* Herzog & Perss. (Heinrichs *et al*., 2000) and *Platyhypnidium torrenticola* (Ochyra, C.Schmidt & Bültmann) Ochyra & Bednarek-Ochyra (Werner *et al*., 2007). On the other hand, incongruence between phenotypic and molecular phylogenetic inferences was studied in depth in the island endemic *Leptodon corsicus* Enroth, A.Sotiaux, D.Quandt & Vanderp., and it was suggested that the time since speciation might not have been long enough for the sorting of alleles to be complete (Sotiaux *et al*., 2009), as an example of 'budding speciation' (see Funk & Omland, 2003, for a review). This suggests that many bryophyte species have failed to diversify at the molecular level in Macaronesia (Vanderpoorten *et al*., 2008; Stech *et al*., 2011; Hutsemékers *et al*., 2012; Patiño *et al*., 2013a). However, there are also molecular studies that have revealed the opposite pattern of genetic variation. An example is the liverwort *Radula lindenbergiana* Gottsche ex Hartm., present in all the Macaronesian archipelagos, which exhibited a high diversification in haplotypes (Laenen *et al*., 2011), comparable to that reported for many angiosperm groups at the species (Carine, 2005) and molecular (Schaefer *et al*., 2011) levels.

The moss genus *Isothecium* Brid. (Lembophyllaceae, Hypnales, Bryophyta) is notably diversified in Macaronesia, where four species are recognized, two of which show restricted distribution areas: one is endemic to Macaronesia [*I. prolixum* (Mitt.) Stech, Sim-Sim, Tangney & D.Quandt = *Echinodium prolixum* (Mitt.) Broth.; Stech *et al*. (2008)] and one is restricted to Macaronesia and the Iberian Peninsula (*I. algarvicum* W.E.Nicholson & Dixon). The remaining two species, *I. alopecuroides* (Lam. ex Dubois) Isov. and *I. myosuroides* Brid., are widely distributed in Europe including Macaronesia, northern Africa and North America (Schofield, 2014; Ros *et al*., 2013). *Isothecium* is currently well understood in Europe from the morphological point of view, and its taxonomy has remained more or less stable for the last 30–40 years, especially due to the studies of Isoviita (1981) and Hedenäs (1992). However, molecular data have shown that the genus has complex species relationships in Europe, which could be explained by reticulation driven by recurrent exchange of genetic material (Draper, Hedenäs & Grimm, 2007). In this framework, the aim of the present study is to infer: (1) how many genetic entities of *Isothecium* are present in Macaronesia; (2) whether the genetic entities are congruent with the taxa currently recognized based on morphology; and (3) whether the number of Macaronesian endemic *Isothecium* species has been correctly assessed.

MATERIAL AND METHODS

Selected taxa

To assess the number of genetic entities of *Isothecium* present in Macaronesia and if they are congruent with the taxa that can be recognized based on morphology, we combined a molecular investigation with a morphological study. For the molecular approach, we focused the selection of material on the *Isothecium* species endemic or nearly endemic to Macaronesia. We selected 32 specimens of *I. prolixum* that cover most of its distribution area: the Azorean (Gabriel *et al*., 2005) and Madeiran archipelagos (Sérgio *et al*., 2008). For *I. algarvicum*, we selected 20 specimens, also covering its distribution area: the Canary Islands (González-Mancebo *et al*., 2008), Madeira (Sérgio *et al*., 2008) and the south-western Iberian Peninsula (Sérgio & Carvalho, 2003). According to current knowledge, *I. alopecuroides* (seven specimens included) and *I. myosuroides* [15 specimens, including

I. alopecuroides var. *brachythecioides* (Dixon) C.E.O.-Jensen] are the closest relatives of *I. algarvicum* (Draper *et al*., 2007) and *I. prolixum*, respectively (Stech *et al*., 2008). To obtain a phylogenetic framework, we also included other *Isothecium* spp. that do not occur in Macaronesia: the European *I. holtii* Kindb. (Ros *et al*., 2013); the North American *I. stoloniferum* Brid., *I. cristatum* (Hampe) H.Rob. and *I. cardotii* Kindb. (Schofield, 2014); and the Asian *I. subdiversiforme* Broth. (Iwatsuki, 2004). *Isothecium* belongs to the family Lembophyllaceae (Quandt *et al*., 2009), which, according to Huttunen *et al*. (2012), is sister to Leucodontaceae, and these two are sister to Neckeraceae. Thus, we also included members of Lembophyllaceae [*Camptochaete arbuscula* (Sm.) Reichardt, *Lembophyllum divulsum* (Hook.f. & Wilson) Lindb., *Rigodium implexum* Kunze ex Schwägr. and *Weymouthia mollis* (Hedw.) Broth.], Leucodontaceae {*Nogopterium gracile* (Hedw.) Crosby & W.R.Buck [= *Pterogonium gracile* (Hedw.) Sm.; Crosby & Buck, 2011]} and Neckeraceae {*Alleniella complanata* (Hedw.) S.Olsson, Enroth & D.Quandt [= *Neckera complanata* (Hedw.) Huebener; Olsson *et al*., 2011], *Leptodon smithii* (Hedw.) F.Weber & D.Mohr (used to root the tree), *Neckera cephalonica* Jur. & Unger and *Neckera pumila* Hedw.}.

Complementary to the molecular analyses, we studied the morphological features of all the *Isothecium* specimens included in the molecular section plus additional specimens of the species occurring in Macaronesia, which constitute our ingroup. In total, we analysed 44 specimens of *I. algarvicum*, 68 of *I. prolixum* and ten of *I. prolixum* that deviate morphologically from the rest and that correspond to the high-elevation form discussed below. For the remaining taxa included in the molecular analyses, we evaluated a limited selection of specimens, to complete the comparison of the main morphological traits that allow the identification of the endemics. A complete list of the scored specimens is included in Appendix 1, indicating those included in the molecular analyses.

DNA extraction, PCR and sequencing

Of the 257 sequences included in the analyses, 43 were downloaded from GenBank (for accession numbers and references, see Appendix 1) and 214 were newly generated for this study. Total DNA was extracted from 1-cm tips of the stems using the NaOH method of Werner, Ros & Guerra (2002) or the Plant DNeasy Mini Kit from Qiagen. PCR was performed in an Eppendorf Mastercycler using PuReTaq Ready-To-Go PCR Beads (GE Healthcare) in a 25-μL reaction volume according to the manufacturer's instructions. Three molecular regions were amplified, one from the nuclear genome (internal transcribed spacers 1 + 2, ITS) and two from the plastid (*trnG* and *trnL-trnF*). In all cases the PCR programmes given below were initiated by a melting step of 5 min at 95 °C and followed by a final extension period of 8 min at 72 °C. For the ITS the PCR programme employed was 35 cycles of 30 s at 95 °C, 30 s at 52 °C and 105 s at 72 °C, with the primers 18F-Iso (Draper *et al*., 2011) and 25R (Stech & Frahm, 1999). Occasionally, ITS1 and ITS2 were separately amplified, with the primers 18S/5.8R (ITS1) and 5.8F/25R (ITS2), designed by Spagnuolo *et al*. (1999; 18S) and Stech & Frahm (1999; the rest). For the plastid intron *trnG*, 35 cycles of 30 s at 95 °C, 40 s at 51 °C and 90 s at 72 °C were employed, with the primers trnGf-Leu (Stech *et al*., 2011) and trnGr (Pacak & Szweykowska-Kulinska, 2000). The number of cycles for amplifying the *trnG* intron was increased to 40 in samples where DNA was difficult to amplify. Finally, for the plastid *trnL-trnF* region (*trn*L_{UAA} intron and *trn*L_{UAA}-*trn*F_{GAA} spacer), 35 cycles of 30 s at 95 °C, 45 s at 51 °C and 90 s at 72 °C were employed, with the primers c and f (Taberlet *et al*., 1991). After visualization on 1% agarose gels, successful amplifications were cleaned with the GenElute PCR Clean-Up kit (Sigma-Aldrich Biotechnology). The amplification primers were used in the sequencing reactions with the Big Dye sequencing kit and the sequencing products separated on an ABI-Prism 3700 at Secugen (http://www.secugen.es).

Sequence editing, alignment and phylogenetic analysis

Nucleotide sequences were edited and assembled for each DNA region in PhyDE v0.9971 (Müller *et al*., 2006). The assembled sequences were manually aligned according to the criteria of Kelchner (2000). Regions of incomplete data at the 5′ and 3′ ends of the sequences were excluded from subsequent analyses (ITS1 5′, 77 positions; ITS2 3′, 67; *trnG*, 71/57; *trnL-trnF*, 25/23). The *trnL-trnF* spacer presents a small loop with a 3-nt inversion. Quandt & Stech (2004) showed that this inversion changes at the population level, which can be highly problematic in phylogenetic reconstructions [Quandt, Müller & Huttunen (2003), but also see discussion in Borsch & Quandt (2009) and Hedenäs (2011)]. We found no variation correlated with morphology in this inversion, and it was excluded from the analyses.

Phylogenetic reconstructions were made on the basis of three different analytical methods: neighbour joining (NJ), maximum parsimony (MP) and Bayesian inference (BI), using the programs Mega 6 (Tamura *et al*., 2013) for NJ, TNT 1.1 (Goloboff, Farris & Nixon, 2003) for MP and MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003;

Ronquist *et al*., 2012) for BI. The substitution model selected in Mega was maximum composite likelihood, including transitions and transversions, with uniform rates and homogeneous patterns. The swapping algorithm selected in TNT was tree bisection reconnection (TBR), with ten trees held in memory. All characters were equally weighted. Clade support in NJ and MP analyses was assessed via non-parametric bootstrapping with 1000 replicates. For all generated MP trees the consistency index (CI), retention index (RI) and tree length were calculated.

The nucleotide substitution models used for the BI analysis were selected for each partition with jModeltest 2.1.4 (Posada, 2008; Darriba *et al*., 2012) based on Akaike and Bayesian information criteria: HKY+I+G for ITS1, K80 for 5.8S, GTR+G for ITS2, GTR+G for *trnG* and HKY+G for *trnL-trnF*. The BI analysis used one cold and three incrementally heated Monte Carlo Markov chains (MCMC) on two simultaneous runs. MCMC runs continued until the standard deviation of split frequencies was below 0.01 (5 000 000 generations), with one tree sampled every 1000th generation, each using a random tree as a starting point and a temperature parameter value of 0.2 (the default in MrBayes). The first 25% of the total sampled trees of each run were discarded as burnin, to achieve the MCMC log-likelihoods that had become stationary and converged.

Indels in non-coding regions are sometimes difficult to assess (Kelchner, 2000). In *Isothecium*, part of the ITS region includes numerous indels, therefore to determine the effect of their inclusion, all the analyses were run with the indels considered as missing information and with insertions and deletions coded as potentially informative characters. Indels were coded in SeqState (Müller, 2004), using the simple indel coding strategy (Simmons & Ochoterena, 2000): all gaps are coded as separate presence/absence characters, but whenever gaps from different sequences may be a subset of other gaps, sequences are coded as inapplicable for the gap character being coded. As the indel partition is binary, it was analysed in MrBayes under an F81-like model, with an ascertainment (coding) bias selected as variable, as recommended by Ronquist, Huelsenbeck & van der Mark (2005). The rest of the parameters of the analyses were the same as those indicated for the data sets without coded indels.

Low resolution in phylogenetic reconstructions can sometimes be caused by incongruence or conflicts in the molecular data sets that lead to different equally possible solutions (e.g. Huson & Bryant, 2006). To check if this occurs in our data we reconstructed a phylogenetic network based on the neighbour-net method (NN; Bryant & Moulton, 2004), using the program SplitsTree4, version 4.13.1 (Huson & Bryant, 2006).

Morphological study

To assess if the genetic entities recovered in *Isothecium* correspond to taxa that could be recognized based on morphology, we carried out a morphological study of all the *Isothecium* species included in the molecular analyses. For the species endemic or nearly endemic to Macaronesia, we scored > 100 morphological characters of the sporophyte and the gametophyte. This includes all characters that have been proved to be taxonomically useful in *Isothecium* or related genera. Thus, we have been able to provide a complete morphological description of a new species (see below). In addition, the knowledge of morphology has been useful to decide whether molecularly deviating specimens corresponded to misidentifications.

In addition, we selected 16 diagnostic gametophyte and sporophyte characters (Table 1) and we scored these for the non-endemic *Isothecium* taxa included in the molecular analyses (Appendix 1). These 16 diagnostic features were coded and used to reconstruct morphology-based phylogenetic trees for *Isothecium*, with MP and BI, using the same programs indicated for the molecular phylogenetic analyses. The matrix of coded morphological characters is available upon request. The MP phylogenetic tree was built with the same parameters and options described for the molecular phylogenetic analysis. In the BI phylogenetic tree, the options selected were also similar to those in the molecular phylogenetic analysis, with the exception of the model selected (JC-like with equal rates for standard data, characters treated as unordered), and the number of generations (1 000 000 to reach a standard deviation of split frequencies below 0.01).

RESULTS

Molecular sequence variation

Length variation of the three regions ITS1–5.8S–ITS2, *trnG* and *trnL-trnF* is shown in Table 2, with the information content of each data partition. As no incongruence in terms of well-supported clades was observed in separate analyses of the three molecular regions (trees are supplied as Supporting Information, Fig. S1), combined analyses were performed. The combined data matrix had a total length of 1828 bp, with 189 variable sites (108 potentially parsimony-informative, 49 within the ingroup). Simple indel coding increased the number of potentially parsimony-informative characters, with 28 characters in the nuclear data set (three for the ingroup) and seven in the plastid data sets (six for the ingroup). The trees obtained from the analyses of the combined data matrix including the indels resolved more clades and with higher support than those resulting from the analyses of the different regions separately or without

Table 1. Main morphological traits to distinguish the *Isothecium* species included in the study; leaf characters refer to stem leaves except in the column comparing branch leaves with stem leaves

Species	Plant size	Widest part of leaf (leaf shape)	Leaf apex shape	Leaf curvature	Costa number	Costa length	Costa width near base (μm)	Costa prorate or papillose	Mid-leaf cell size (μm)	Mid-leaf cell surface	Alar group size	Alar group shape
I. algarvicum	Small to medium	Well below mid-leaf (ovate to triangular ovate)	Gradually acuminate or acute	Concave to strongly concave	Single, sometimes forked above	(1/2)3/5–4/5 of leaf length	31–73	Rarely ending in a spine	10–44(54) × 5–9 μm	Smooth or with scattered prorate cells on back above	Large, extends 1/4–1/3 or more upwards along margin	Triangular
I. alopecuroides	Medium	Below mid-leaf (ovate oblong)	Gradually rounded with acute apiculous, acute or obtuse	Strongly concave	Single or sometimes forked	3/5–4/5 of leaf length	67–105	Sometimes ending in an abaxial spine	25–76 × 6–10 μm	Distally prorate on back above	Small, 1/20–1/10 upwards along margin	Quadrate or shortly oblong
I. cardotii	Robust plants	Well below mid-leaf (broadly to narrowly triangular)	Abruptly or mostly gradually acuminate, rarely acute	Concave, sometimes falcate	Single, sometimes forked	Up to 3/4 of leaf length	84–158	Sometimes ending in an abaxial spine	36–94 × 5–10 μm	Smooth or occasional to scattered prorate cells on back above	Small, extending less than 1/10 upwards along margin	Triangular
I. cristatum	Medium	Below mid-leaf (broadly ovate)	Gradually acuminate	Concave, sometimes falcate	Single	1/2–2/3 of leaf length	59–103	Ending in a stout spine on back of leaf	21–53 × 6–11 μm	Smooth or with scattered spine-like prorations on back above	Large, extends 1/4–2/5 upwards along margin	Narrowly triangular
I. holtii	Medium	Below mid-leaf (ovate to cordate triangular)	Gradually acuminate or shortly acuminate	Concave	Single	1/2–4/5 of leaf length	63–126	Smooth, or occasionally ending in small abaxial spine	23–68 × 5–9 μm	Smooth or distally prorate cells on back in the uppermost part	Small, extending less than 1/6 upwards along margin	Ovate or triangular ovate
I. montanum	Medium to large	Below mid-leaf (ovate, broadly ovate or rounded triangular)	Gradually shortly acuminate	Slightly concave to almost plane	Single, sometimes branched or forked above	1/2–3/4 of leaf length	82–130	Smooth	17–46(50) × 4–10 μm	Smooth	Not differentiated from other basal cells or diffuse and not ascending upwards along margin	Not differentiated from other basal cells or round
I. myosuroides	Medium	Well below mid-leaf (cordate or broadly cordate)	Abruptly narrowed to longly and narrowly acuminate	Concave	Single, generally bifurcated above	2/3 of leaf length	56–84	Apical cell in some nerves prorate on back of leaf	12–57 × 6–9 μm	Occasional to scattered prorate cells on back	Small, extending less than 1/5 upwards along margin	Triangular
I. myosuroides var. *brachythecioides*	Medium to large	Below mid-leaf (ovate-oblong to broadly cordate)	Gradually or suddenly narrowed to narrowly acuminate	Concave	Single or often branched or forked, sometimes from shortly above insertion	(1/6)1/3–2/3 of leaf length	63–105	Smooth	23–70 × 6–10 μm	Smooth	Small, extending less than 1/8 upwards along margin	Rounded quadrate or transversely elongated
I. prolixum	Medium to large	Below mid-leaf (broadly lanceolate to rarely cordate triangular)	Gradually (rarely suddenly) narrowed to narrow or broad acuminate point	Concave	Single	Over 2/3 of leaf length	84–150	Smooth	(9)12–51(63) × 5–11 μm	Smooth	Not differentiated from other basal cells	Not differentiated from other basal cells
I. subdiversiforme	Medium to large	Below mid-leaf (ovate to ovate triangular)	Gradually acuminate or broadly acuminate	Strongly concave	Single or forked	1/2–3/4 of leaf length	63–90	Smooth or ending in a small spine on back	16–59 × 4–7 μm	Smooth, or sometimes with scattered prorate cells on back above	Small, extending less than 1/10 upwards along margin	Rounded or shortly transversely triangular

Table 1. *Continued*

Species	Branch leaf differentiation from stem leaf	Seta length (mm)	Capsule orientation	Exostome PPL ridges
I. algarvicum	Smaller and narrower, prorate cells more numerous and nerve sometimes ending in spine. Proximal leaves broad, sometimes broadly rounded at apex.	11–13 mm	Horizontal or inclined	Normal
I. alopecuroides	Smaller and narrower, more strongly denticulate. Proximal leaves semi-orbicular, obtuse.	10–15 mm	Erect	Reduced
I. cardotii	Smaller and narrower, more longly acuminate, sometimes twisted, more strongly denticulate above; prorations of lamina cells and costa spine sometimes more prominent.	10–20 mm	Inclined	Rather narrow teeth with PPL ridges lower than in normal unspecialized peristomes
I. cristatum	Smaller and narrower, dorsal spine-like prorations more common. Proximal leaves suborbicular, with rounded to broadly obtuse apex.	10–14 mm	Erect or inclined	Normal
I. holtii	Smaller, more shortly acuminate or acute, costa sometimes ending in dorsal spine, prorate cells more common. Proximal leaves semi-orbicular with apex broadly rounded.	11–17 mm	Horizontal to almost inclined	Normal
I. montanum	Smaller. Proximal leaves with apex broadly acute to obtuse.	Not found	Not found	Not found
I. myosuroides	Smaller and narrower. Proximal leaves semi-orbicular, obtuse.	15–18 mm	Inclined to almost horizontal due to seta curvature	Normally developed
I. myosuroides var. *brachythecioides*	Smaller, more strongly denticulate above, sometimes with prorate cells on back and above and with costa ending in dorsal spine. Proximal leaves broadly ovate or semi-orbicular, with broadly acute to rounded apex.	8–10 mm	Erect or inclined due to seta curvature	Lower than in normal unspecialized peristomes
I. prolixum	Smaller and narrower, apex obtuse to broadly acuminate.	15–20 mm	Erect to slightly inclined or horizontal	Normal
I. subdiversiforme	Smaller and narrower, with acute or shortly acuminate apex. Proximal leaves suborbicular, sometimes rounded at apex.	8–13 mm	Erect, inclined or horizontal due to seta curvature	Lowered

PPL, Primary Peristomial Layer

Table 2. Actual length variation, number of variable and potentially informative sites and substitution models for ITS, *trnG* and *trnL-trnF* sequences used in the study

		Nuclear data	Plastid data	
	Species	ITS1–5.8S–ITS2	*trnG*	*trnL-trnF*
Length variation	*Alleniella complanata*	678	490	373
	Camptochaete arbuscula	642	–	376
	Isothecium algarvicum	646	497–508	373
	Isothecium alopecuroides	646	497–509	373
	Isothecium cardotii	656	490	373
	Isothecium cristatum	647	490	381
	Isothecium holtii	657	490	–
	Isothecium myosuroides	657–658	490	373–416
	I. myosuroides var. *brachythecioides*	656–657	490	373
	Isothecium prolixum	657	490	373
	Isothecium 'prolixum' montanum	657	490	373
	Isothecium stoloniferum	649	497	377
	Isothecium subdiversiforme	639	490	392
	Lembophyllum divulsum	643	–	376
	Leptodon smithii	677	490	373
	Nogopterium gracile	647	502	376
	Neckera cephalonica	646	490	372
	Neckera pumila	645	490	373
	Rigodium implexum	637	–	376
	Weymouthia mollis	652	–	376
Variable sites		111	49	29
Variable sites (ingroup)		58	26	12
Potentially informative sites		68	25	15
Potentially informative sites (ingroup)		28	14	7
Gap sites		72	12	6
Gap sites (ingroup)		10	11	6
Potentially informative gap sites		28	4	3
Potentially informative gap sites (ingroup)		3	4	2
Positions in data matrix		1–300 / 301–471 / 472–761	762–1293	1294–1743
Substitution model		HKY+I+G / K80 / GTR+G	GTR+G	HKY+G

indels coded. Hereafter we present the tree and network obtained from the analyses of the combined data matrix with the indels included, although we also indicate support values of the clades without including the indels.

Phylogenetic analyses

As the main aim of our study was to identify how many genetic entities of *Isothecium* are present in Macaronesia, and if these are congruent with the taxa recognized based on morphology, we initially ran the phylogenetic analyses based on the molecular evidence alone. All the analyses recovered trees with congruent topologies for the ingroup taxa, independently of the applied method (BI, MP or NJ), although the best resolution was obtained using BI and with indels coded [see Fig. 1, in which posterior probability (PP) and bootstrap (BS) support values are indicated for the different clades recovered]. We hereafter refer to the support values in the text following the scheme (PP/PPindels/BS in MP/BSindels in MP/BS in NJ/BSindels in NJ), to show if the grouping was supported by BI, MP or NJ analyses. The MP analysis retained 165 trees [length 513, consistency index (CI) = 0.817, retention index (RI) = 0.927].

The present analyses resolve *Isothecium* as monophyletic in a well-supported clade (Fig. 1A, ingroup, 1/1/93/91/–/–). In this clade, the species occurring in Macaronesia are were placed in two clades, one holding the endemic *I. algarvicum* with *I. alopecuroides* (Fig. 1B, clade A; –/0.77/95/–/–/–) and the second comprising the endemic *I. prolixum* with *I. myosuroides*, which are together sister to *I. holtii* (Fig. 1B, clade C; 1/1/100/95/71/69). In clade A, *I. alopecuroides* forms an unsupported subclade (clade B) on the basis of a single mutation in the *trnG* region (Table 3), whereas *I. algarvicum* is not resolved into a single clade. Similarly, the studied samples of *I. prolixum* are divided into four clearly separated groups in clade C, which

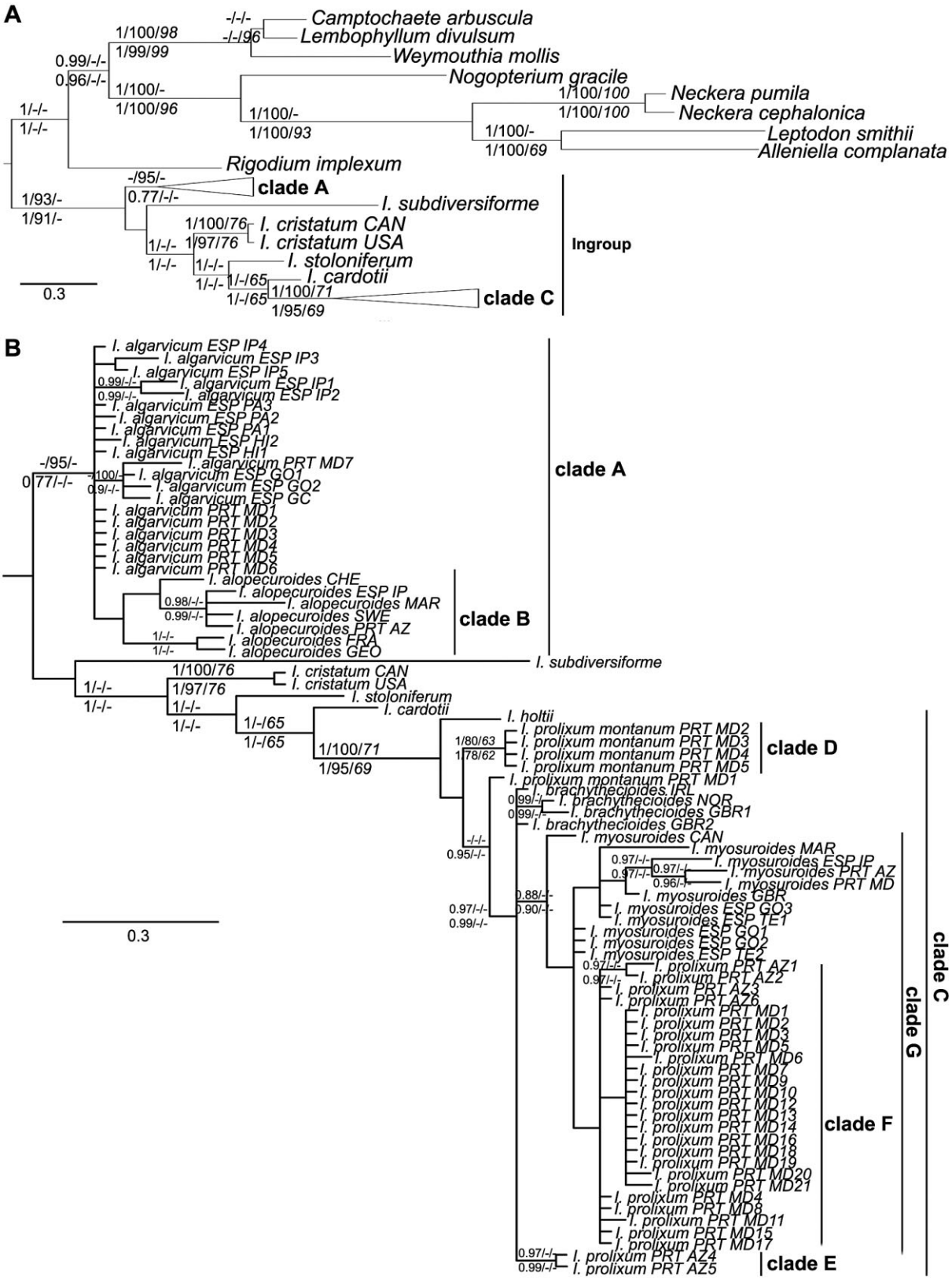

Figure 1. See caption on next page.

Figure 1. Consensus phylogram based on Bayesian inference resulting from the analysis of the matrix combining ITS, *trnG* and *trnL-trnF* data sets, including a simple coded indel matrix. Numbers above the branches indicate node supports considering gaps as missing data, and numbers below the branches indicate node supports considering gaps as informative characters. The first figure corresponds to posterior probability (PP) according to BI, the second corresponds to bootstrap support (BS) according to MP, and the third to BS according to NJ. Values shown are those > 60. The tree was rooted to the node between the ingroup and the outgroup taxa. A, summary of the tree with all the taxa included in the study. B, detailed tree for the ingroup taxa (the genus *Isothecium*). Abbreviations for the location data correspond to Spain (ESP), Iberian Peninsula (IP), La Palma (PA), El Hierro (HI), La Gomera (GO), Gran Canaria (GC), Tenerife (TE), Portugal (PRT), Madeira (MD), Azores (AZ), Switzerland (CHE), Morocco (MAR), Sweden (SWE), France (FRA), Georgia (GEO), Canada (CAN), United States (USA), Ireland (IRL), Norway (NOR) and Great Britain (GBR); numbers correspond to the samples listed in the Appendix.

Table 3. Genetic variation differentiating clades B, D, E and F

		Nuclear data	Plastid data	
Clade	Variations	ITS1–5.8S–ITS2	*trnG*	*trnL-trnF*
Clade B *I. alopecuroides*	1 mutation	–	Position 879, retain ancestral A (other *Isothecium* spp.: G)	–
Clade D *I. montanum* except MD1	7 mutations	Position 72, retain ancestral T (C in *I. prolixum* and *I. myosuroides s.l.*) Position 293, retain ancestral A (G in *I. prolixum*, *I.montanum* MD1, and *I. myosuroides s.l.*) Position 297, T (all the other samples included in the study, from all taxa including *I. montanum* MD1: C) Position 581, A instead of C (shown by most of *I. prolixum* and *I. myosuroides s.l.* samples) Position 670, T (G in the majority of the other *I. prolixum* and C in most *I. myosuroides s.l.* samples).	Position 1064, G (all the other samples from all taxa included in the study: A; this position is lacking in MD1)	Position 1458, retain ancestral A (*I. prolixum*: C)
Clade E *I. prolixum* AZ4 and AZ5	4 mutations	Position 178, retain ancestral G (other *I. prolixum* and *I. myosuroides s.l.* samples: A) Position 188, retain ancestral A (other *I. prolixum* and *I. myosuroides* samples: C) Position 581, A instead of C (shown by other *I. prolixum* and *I. myosuroides s.l.* samples) Position 670, T (G in the other *I. prolixum* and C in most *I. myosuroides s.l.* samples)	–	–
Clade F *I. prolixum* except AZ4 and AZ5	1 mutation	Position 670, G (T in *I. montanum* and *I. myosuroides* var. *brachythecioides*; C in most *I. myosuroides s.s.* samples)	–	–

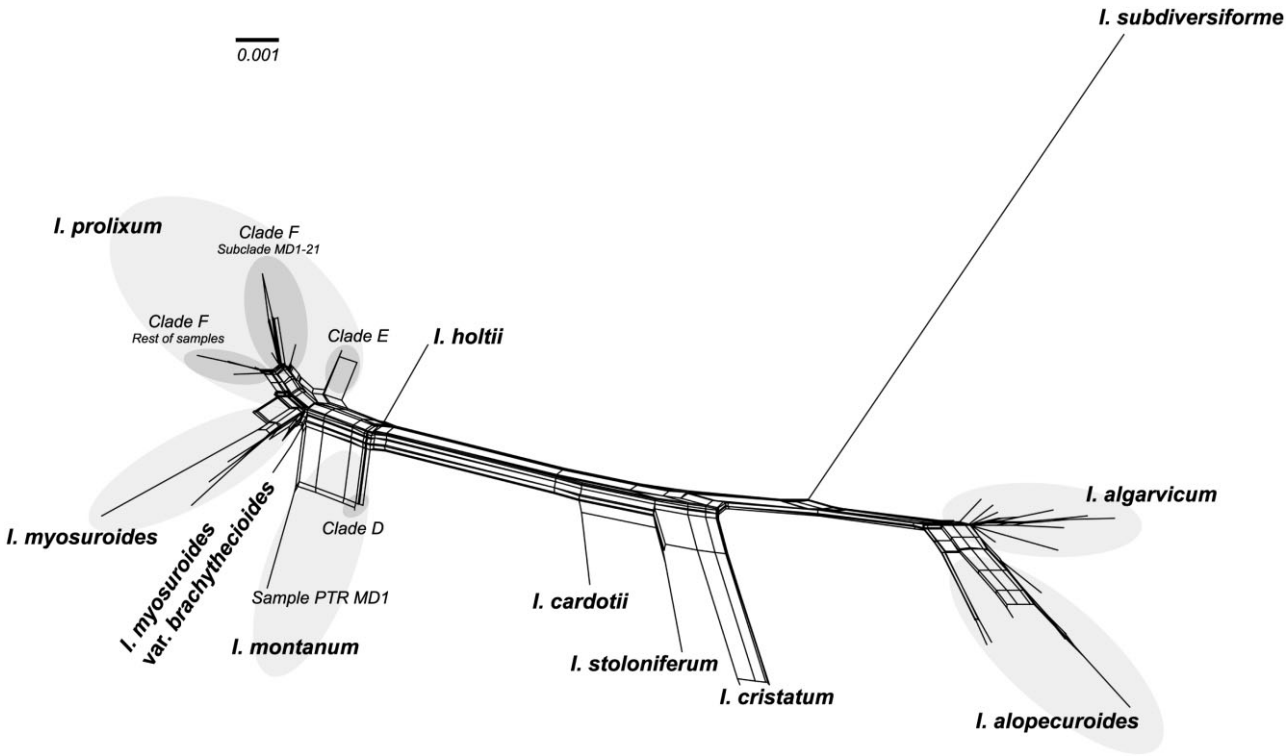

Figure 2. Consensus phylogenetic network (neighbour-net) resulting from the analysis of the molecular matrix combining the ITS, *trnG* and *trnL-trnF* data sets. Clades D–F correspond to those identified in Figure 1. Sample PRT MD1 is listed in the Appendix.

correspond to two morphotypes after revision of their morphological characters (see Discussion). The first morphotype (clades E and F, distinguished by four mutations or a single mutation in ITS, respectively; Table 3) corresponds to morphologically typical *I. prolixum*. The second morphotype, hereafter named *I. montanum*, clusters in two groups (clade D and sample PRT MD1) located between *I. holtii* and the rest of clade C. Clade D is well supported (1/1/80/78/63/62) on the basis of five mutations in the ITS region, one in *trnG* and one in *trnL-trnF* (Table 3). Sample PRT MD1 shows the same morphological features as the specimens clustered in clade D, but lacks two of the mutations in the ITS region, and the information regarding the *trnG* region is missing. In *I. myosuroides*, all four samples of var. *brachythecioides* occupy an early-branching position with respect to var. *myosuroides* plus *I. prolixum p.p.* (clade G, almost supported by PP: 0.88/0.90/–/–/–/–), but further relationships remain unsupported.

The consensus phylogenetic network reveals that several relationships among specimens are possible for our data, which is shown in Figure 2 as multiple alternative splits (box-like portions). Overlapping split patterns indicate different possible evolutionary connections. These splits can be correlated in particular to such nodes with low resolution in the phylogram. Thus, samples of *I. prolixum*, *I. myosuroides*, *I. myosuroides* var. *brachythecioides* and *I. montanum* (clade C in Fig. 1) are closely related in the left edge of the network by different possible evolutionary paths. As an example, sample *I. montanum* MD1 is related to the samples of *I. myosuroides* var. *brachythecioides*, but also to the other samples of *I. montanum* (noted as clade D). Similarly, *I. prolixum* is not recovered in a single well-supported clade in the phylogram (Fig. 1), although all *I. prolixum* samples are placed nearby in the phylogenetic network (Fig. 2). The three groups of *I. prolixum* samples, clade E and both subclades of F (samples MD1–21 and remaining samples), are connected through different possible evolutionary paths, showing a box-like structure where they can be equally related to each other and to *I. myosuroides*. The same kind of splits can be found for samples of *I. algarvicum* and *I. alopecuroides* (clade A in Fig. 1), which are closely related in the right edge of the network (Fig. 2). Within these two morphotaxa alternative splits are especially abundant among *I. alopecuroides* samples, corresponding to the unsupported clade B in Figure 1. It is, however, remarkable that the samples of the

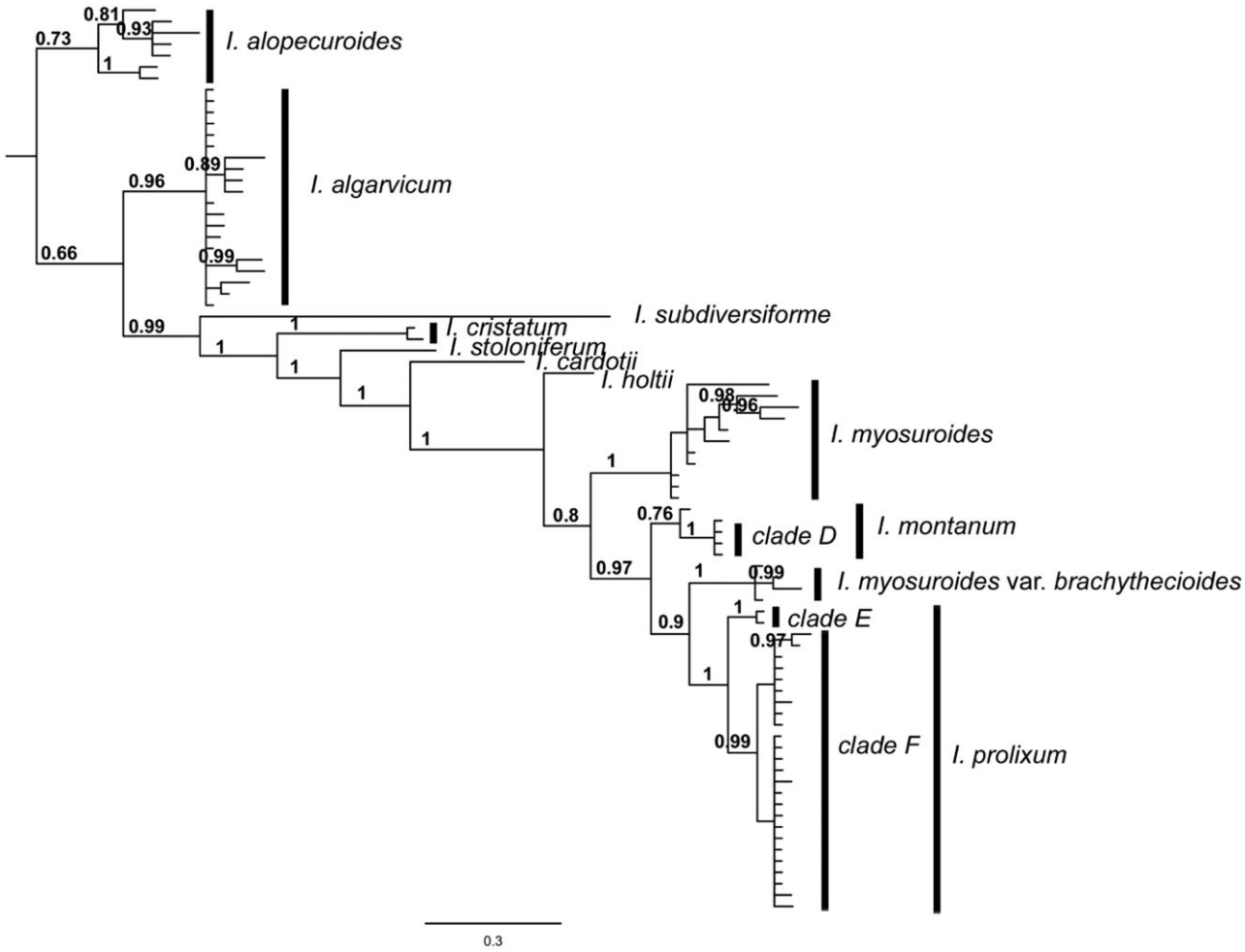

Figure 3. Consensus phylogram for the ingroup taxa (*Isothecium*) based on Bayesian inference resulting from the analysis of the matrix combining ITS, *trnG* and *trnL-trnF* data sets, including a simple coded indel matrix, and a morphological matrix including 16 coded characters. Numbers above the branches indicate node support (PP). Clades D–F correspond to those identified in Figure 1.

different morphotaxa are not intermixed, and separate groups containing all the samples of each taxon could be delimited (Fig. 2, light shadowed ellipses).

The 16 diagnostic morphological characters that can be used to distinguish the different *Isothecium* taxa of our ingroup do not allow recovery of a resolved phylogenetic tree based on morphology alone (unresolved tree not shown). Therefore, the following discussion about the congruence of the genetic and morphological entities of *Isothecium* present in Macaronesia is based mainly on the position of the different morphotaxa in the molecular phylogenetic reconstruction. However, when all our data are analysed together (molecular data including indels plus morphological characters), the 16 morphological characters provided enough additional evidence to recover all the morphotaxa in separate moderately to strongly supported clades (Fig. 3). The topology of the tree recovered with all the available data combined is overall congruent with the topology of the tree based on the molecular data alone (Fig. 1), except for the relationships inside clades A and C as noted in Figure 1. The morphological evidence does not support clade A, and instead places *I. alopecuroides* in an early branching position, in a poorly supported clade (PP 0.73). The molecular evidence alone does not resolve *I. algarvicum*, but when the morphological data are taken into account, *I. algarvicum* is resolved in a well-supported clade (PP 0.96), next to *I. alopecuroides*. Regarding clade C in Figure 1, the inclusion of the morphological data provides support for the delimitation of four morphotaxa in separate clades: *I. myosuroides* (PP 1); *I. montanum* (with poor support, PP 0.76, for the clade containing all the samples, but strong support, PP 1, for subclade D); *I. myosuroides* var. *brachythecioides* (PP 1); and

I. prolixum (PP 1). Within the last, the two clades E (PP 1) and F (PP 0.99) that were recovered on the basis of the molecular data are strongly supported as sister clades (PP 1).

DISCUSSION

When using standard tree-based phylogenetic methods, several species in *Isothecium*, which are morphologically well characterized, are not clearly separated based on the molecular data employed here, despite the combined use of three supposedly rapidly evolving non-coding molecular markers and the inclusion of the indels. This finding is in line with an increasing number of phylogenetic studies (see Vanderpoorten & Shaw, 2010, for a review), whereas in other cases molecular data confirmed bryophyte species circumscriptions that were ambiguous based on morphology alone (e.g. Stech *et al*., 2013; Lang & Stech, 2014).

The possible sources of incongruence between morphology and molecular data have been classified into methodological and biological (Dávalos *et al*., 2012). Among the methodological sources, misidentification of samples may be a common cause of incongruence. In this case, this is unlikely, because all the molecularly deviating specimens with uncertain positions in the phylogram were morphologically revised after the phylogenetic reconstruction, and misidentification could be ruled out except for the specimens named *I. montanum* (see below). In addition, all the samples of the same morphotaxon are grouped in the neighbour-net.

A second possible methodological explanation for the incongruence could be inadequate sampling, either regarding ingroup or outgroup taxa, or selection of genes that include too few informative characters (Vanderpoorten & Shaw, 2010; Dávalos *et al*., 2012). Mutation rates in close lineages of bryophytes may be different for some regions (e.g. Hedenäs, 2009), and it is therefore complex to establish the level of molecular variation that accomplishes taxonomic variation in different groups. However, the employed regions (*trnG*, *trnL-trnF* and ITS) apparently provide sufficient variation to infer species circumscriptions in *Isothecium*, as some of the taxa included in this study show well-separated haplotypes, such as *I. holtii*, *I. cardotii*, *I. stoloniferum*, *I. cristatum*, *I. subdiversiforme* or the well-supported clades A and C of Figure 1, even when the different genomes are treated separately (Supporting Information, Fig. S1).

One last possible methodological shortcoming could be the choice of the analytical method (Dávalos *et al*., 2012), including poor model specification. We have used four different methods (BI, MP, NJ and NN) for the analyses of the molecular data, and all four recovered similar topologies for the ingroup, although many of the clades lacked support. The probability of an inadequate choice of the analytical method is therefore discarded. Moreover, within the four methods used, the best resolution and support was obtained with BI, which probably indicates that the substitution models were correctly assessed.

We therefore suggest that if molecular trees are not able to resolve all the *Isothecium* lineages defined by morphology, this might be of biological origin. In some cases, incongruence has been explained by adaptive convergence (e.g. Shaw & Allen, 2000). In such examples, clades that include apparently unrelated taxa reflect geographical or ecological patterns. A deep analysis of the ecological traits and geographical origin of the samples included in this study (Appendix 1) does not reflect any of these patterns for the specimens included in clades A and C.

The different degree of molecular and morphological demarcation inferred in the present study for the focal species could ultimately have arisen from incomplete lineage sorting, especially if taxa have diverged recently. It is well established that in the initial phases of multiple speciation, gene trees may not reflect the actual species tree (see Avise, 2000, and references therein). For instance, when two large populations of one species evolve into two species, an initial polyphyletic gene tree is probable. However, if the starting point for a new species is a small isolated population, a paraphyletic gene tree pattern is more likely. In this sense, the use of monophyly as the only criterion for species recognition can result in a species concept that does not reflect the actual speciation process (Zander, 2007), for example in cases of 'budding speciation' (Vanderpoorten & Shaw, 2010). According to Rieseberg & Brouillet (1994), it is additionally difficult to achieve monophyly when there are biogeographical barriers. Funk & Omland (2003) estimated that 23% of all animal species are not monophyletic, whereas Crisp & Chandler (1996) demonstrated that paraphyly occurs in at least *c.* 20% of angiosperms. In bryophytes, several studies have concluded that paraphyletic and polyphyletic relationships at the specific level across different pleurocarpous moss genera could probably be explained by recent divergence and/or rapid morphological evolution in combination with incomplete lineage sorting (Sotiaux *et al*., 2009; Hedenäs, 2011; Carter, 2012; Hedenäs *et al*., 2012), suggesting that the morphology-based species concept should be maintained.

In our study, the evolutionary relationships of the ingroup species *I. algarvicum* vs. *I. alopecuroides* and *I. prolixum* vs. *I. myosuroides* (including var. *brachythecioides*) are not fully resolved when analysed by means of a molecular-evidence phylogram. If the phylogenetic tree is instead represented as a network (Fig. 2), multiple possible evolutionary rela-

tionships (splits) are shown, which potentially result in incongruent relationships in a tree-like representation. Moreover, if the nuclear and plastid sequences are analysed separately, some of the studied samples are located in different positions in the individual trees generated, which can be a source of conflict and one of the causes of the lack of support in trees recovered from the combined data matrix [see as an example the position of the *I. alopecuroides* samples from Morocco (MAR) and Switzerland (CHE), or the sister relationship of *I. prolixum* and *I. holtii* suggested only by the plastid data, in the trees presented as Supporting Information, Fig. S1].

Additionally, another consideration that should be taken into account is that, in some cases, the studied regions are even more variable within than among morphologically defined species. As an example, the genetic distance among the different *I. alopecuroides* haplotypes varies by one to three mutations, plus one insertion. The variation rate observed among the different *I. algarvicum* haplotypes ranges from one to four mutations, plus one insertion. However, there is a single substitution within the studied regions that provides evidence to separate *I. alopecuroides* from *I. algarvicum* (Table 3). A similar pattern holds true for the genetic distance between haplotypes of *I. myosuroides* and *I. prolixum*. However, if the phylogenetic tree is reconstructed in a network, all the samples of each taxon remain closely related and do not intermix with other morphotypes (Fig. 2), although the mentioned differences within the genetic distances can also be seen in the lengths of the branches. Thus, it can be concluded that low resolution obtained in the phylogram within clades A and C is not necessarily due to the inability of the data to define nodes, but to incongruence within different equally possible tree-based evolutionary reconstructions.

The low resolution of the molecular data within clades A and C (Fig. 1) could raise doubts about the validity of the Macaronesian endemics because, as stated in the Introduction, there are several examples of endemics that have been synonymized with more broadly distributed taxa on the basis of their genetic similarity. However, in the case of *Isothecium* there are fixed morphological features that allow the distinction of the different morphotaxa (Table 1), and when these characters are included in the phylogenetic analyses, the different morphotaxa are recovered with moderate to strong support (Fig. 3). In addition, the different taxa occupy mutually exclusive or at least partly non-overlapping habitats and geographical ranges. *Isothecium algarvicum* is both molecularly and morphologically close to *I. alopecuroides*. In contrast, *Isothecium prolixum* is, as suggested by Stech *et al*. (2008), closely related to *I. myosuroides* (Figs 1 and 2). Merging *I. alopecuroides* with *I. algarvicum* or *I. myosuroides* with *I. prolixum* (according to the molecular topologies) would obscure the morphological circumscription of the resulting taxa. By contrast, no morphological characters would support the segregation of *I. alopecuroides*, *I. algarvicum* or *I. myosuroides* into several taxa corresponding to the separate haplotypes already identified by Draper *et al*. (2007). Therefore, we do not propose taxonomic changes for these species.

As indicated above, the lack of resolution of the molecular data to separate the endemics from the more widely distributed taxa can be interpreted as a consequence of recent speciation of *Isothecium* in Macaronesia. In addition, our molecular data suggest that the origin of these two Macaronesian endemics may be explained by at least two independent colonization events, as the species are not closely related to each other, but each is related to one of the two widespread European species (Figs 1 and 2).

Regarding the molecularly deviating samples of *I. montanum*, originally considered to be *I. prolixum*, they show a quite different morphology, especially in stem leaf characters: their leaves are ovate, broadly ovate or rounded triangular, not broadly lanceolate to rarely cordate triangular (as in typical *I. prolixum* leaves), cordate or broadly cordate (as in *I. myosuroides*) or ovate-oblong to broadly cordate (as in *I. myosuroides* var. *brachythecioides*). The costa is well defined and occupies about one-eighth of the leaf base. It is stronger than in *I. myosuroides s.l.*, but not as stout as in typical *I. prolixum*, where it can occupy up to one-quarter of the leaf base, and it is sometimes bifurcate in the upper part (not so in typical *I. prolixum*). These morphological features were already observed and described by Hedenäs (1992) as characteristic of a high-elevation morphological phenotype of *Isothecium* (*Echinodium*) *prolixum*. Hedenäs (1992) indicated that this morphological phenotype could represent a different taxon and pointed out its morphological similarities with *I. holtii*.

The relationship of *I. montanum* and *I. holtii* can also be inferred from our molecular results, as both *I. holtii* and *I. montanum* are found in the early branching part of clade C (Fig. 1), and appear relatively close in the network (Fig. 2). Morphological and molecular differences between these two taxa are provided in Tables 1 and 2. According to our results, *I. montanum* deserves recognition at the species level, as it (1) is morphologically distinct, (2) occurs in a restricted geographical area (Madeira) and (3) grows exclusively on saxicolous habitats at high elevations (at or above 1400 m a.s.l.) within this area, contrary to *I. prolixum s.s*. Below, we provide a formal description of the new species: *Isothecium montanum* Draper, Hedenäs, M.Stech, Lopes & Sim-Sim. It could be argued that according to Figure 1 this new taxon is molecularly paraphyletic. However, the support for

the non-monophyly of *I. montanum* is ambiguous as it is only recovered from the BI analysis with the indels included, whereas it is monophyletic when the morphological characters are taken into account (Fig. 3), with stronger support than the widespread and worldwide recognized *I. alopecuroides*.

Macaronesia has one of the best-known bryophyte floras among oceanic island regions worldwide. However, the delimitation of a new endemic species in *Isothecium*, a genus with such striking diagnostic characters, suggests that current knowledge of diversity and, in particular, endemism may be far from complete across Macaronesia, as recently highlighted for angiosperms (Schaefer *et al*., 2011), and suggested by the description or recircumscription of some other bryophyte species based on molecular evidence (e.g. Aigoin *et al*., 2009; Hutsemékers *et al*., 2012; Hedenäs *et al*., 2014; Werner *et al*., 2014). This could especially apply to plant groups that exhibit limited morphological complexity, such as bryophytes, and that particularly profit from combining several taxonomic methodologies, including morphological and molecular methods. Thus, the present study reinforces the notion that future taxonomic work should focus on integrative approaches for the validation and definition of lineages.

ISOTHECIUM MONTANUM DRAPER, HEDENÄS, M.STECH, LOPES & SIM-SIM, **SP. NOV.** (FIG. 4)

Type MADEIRA, path to Pico Ruivo on rocky humid slopes, 1750 m a.s.l., UTM 28SCB1825, 7 October 2009, *Sim-Sim* (**holotype:** LISU236670!; **isotypes:** S-B177490! and MUB34193!).

Paratypes MADEIRA, Pico Ruivo, highest parts, 1861 m a.s.l., 6 June 1952, *H. Persson* (S-B9263!, LISU236745!, MUB34194!); Pico Ruivo, highest parts,

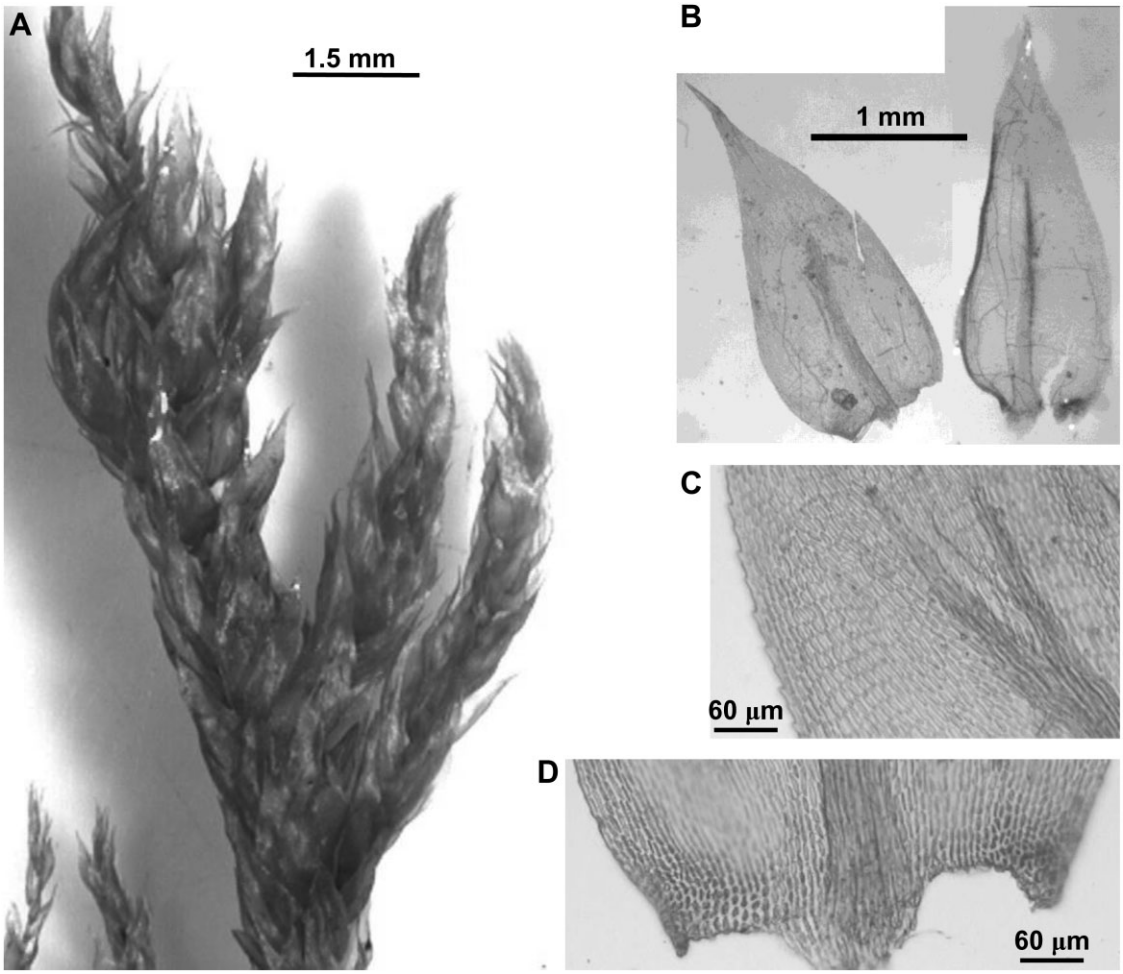

Figure 4. ***Isothecium montanum*** Draper, Hedenäs, M.Stech, Lopes & Sim-Sim **sp. nov.** (Madeira, LISU236671). A, plant habit. B, stem leaves. C, areolation of lamina, margin and costa end in the upper leaf quarter. D, costa and areolation at leaf base.

1861 m a.s.l., June 1952, *H. Persson* (S-B43271!, LISU236746!, MUB34195!); Pico Ruivo, highest parts, 1861 m a.s.l., 5 June 1952, *H. Persson* (S-B9264!); rocks E of Pico Ruivo, 8 km SW of Santana, 1740 m, UTM 28S 31865–362646, N rocks, damp soil along path through upland scrub: *Erica arborea*, etc., 14 October 2009, *G.M. Dirkse*, *H.M.H. van Melick 17* (S-B173706!); Pico Ruivo, rocky slope below *Erica arborea*, 1700 m a.s.l., UTM 28SCB1825, 2 August 2004, *Sim-Sim et al.* (LISU236671!, B200446!; corresponds to PRT MD1); Pico do Arieiro, Casa do Arieiro, *c.* 1550 m a.s.l., 16 June 1952, *H. Persson* (S-B9261!; corresponds to PRT MD2).

Other material studied is listed in Appendix 1.

Plantae mediae vel grandes, obscure flavovirentes. Surculi secundarii suberecti, subdendroidei vel frondosi, sicci cum foliis 1.5–1.8 mm lati, irregulariter pinnatim ramiferi. Folia caulina circa 1.5plo longiora quam latiora, sicca stricta erectaque, ovata vel late ovata vel orbiculato-triangulata, ad apicem breviter acuminatum gradatim decrescentia, leviter concava vel subplana, non vel leviter plicata; margines apicem versus denticulati vel dentati, basin versus subtiliter denticulati vel praecipue prope insertionem integri; costa simplex, longa, prope basin (75–)82–130 μm lata, in parte superiore folii gradatim evanescens vel interdum ramificans vel furcata; laminae cellulae basales 1–4(–5)plo longiores quam latiores; cellulae alares similes vel paulo breviores latioresque, circulariter et excavate aggregatae; cellulae medianae 3–5(–6)plo longiores quam latiores.

Plants medium to large, dull yellowish green, getting brown with age. *Primary shoots* creeping, with small cordate leaves, from erect base patent. *Secondary shoots* suberect, subdendroid or frondose, 1.5–1.8 mm wide with leaves, when dry, irregularly pinnately branched. *Branches* short, 2.0–3.0 (3.5) cm long, usually creeping straight. *Stem* of secondary shoots round or elliptic in transverse section, without or usually with central strand, with basic tissue composed of thin-walled or slightly incrassate cells, and a two- to four-stratose cortex of small and incrassate cells. *Rhizoids* inserted below leaves, especially on primary shoots, red-brown, unbranched or almost so, smooth. *Paraphyllia* not observed. *Pseudoparaphyllia* foliose, oblong, broadly triangular or irregular in shape, obtuse to acuminate, margin often irregular or with large denticles or teeth, lamina cells irregularly rhomboidal to shortly linear. *Axillary hairs* two or three per axil, with two to five upper, hyaline cells, 6.0–11.5 μm wide, basal one or two cells quadrate or rectangular, brown or pale brown. *Stem leaves* (0.6)0.75–1.25 × 1.4–2.0 mm, about 1.5 times longer than wide, straight and erect when dry, straight and patent when moist, ovate, broadly ovate or rounded triangular, gradually narrowed to shortly acuminate apex, narrowed towards insertion, slightly concave to almost plane, not or slightly plicate (especially when dry); margins plane or often partially reflexed on one or both sides, above denticulate to dentate, below finely denticulate or especially near insertion entire, marginal cells slightly shorter than lamina cells further in; costa single, long, ending 55–75% way up leaf, (75)82–130 μm wide near base, smooth, gradually disappearing in the upper part, sometimes branched or forked above, in transverse section plano-convex, in basal part three- to five-stratose, in mid-leaf three- to four-stratose, consisting of homogeneous cells, surface cells on both ad- and abaxial sides similar to adjacent lamina cells or slightly longer; basal lamina cells quadrate to shortly linear, 7–16 × 9–42 μm, one to four (five) times longer than wide, incrassate or strongly so, porose or not, sometimes partly bistratose; alar cells similar to other basal cells or differentiated in a round excavated group of slightly shorter and wider cells, partly or almost entirely bistratose, not or with two to four cells shortly decurrent; median lamina cells elongate-rhomboidal, oblong or linear, (4)5–10 × 17–46(50) μm, three to five (six) times longer than wide, incrassate, slightly porose or not, smooth; apical lamina cells oblong to elongate-rhomboidal, sometimes linear, (7.5) 9.0–15.0 × 22.0–40.0 (45.0) μm, not porose, smooth. *Branch leaves* smaller than stem leaves, 0.4–0.8 × 1.1–1.5 mm, two or three times longer than wide, straight and erect when dry, straight and patent when moist, ovate to lanceolate, slightly concave, not plicate, with margin irregularly denticulate throughout or coarsely denticulate to dentate near apex and finely denticulate to entire near base, proximal branch leaves ovate and obtuse or broadly acute. Specialized vegetative reproductive organs not observed.

Apparently dioicous. *Perigonia* not observed. *Perichaetia* laterally inserted on secondary shoots; inner perichaetial leaves of unfertilized perichaetia smooth, lanceolate, with erect base and patent to spreading acumen, apex acuminate; margin plane, undifferentiated and denticulate above; without costa; lower lamina cells oblong, incrassate or not, not or weakly porose, smooth, upper cells linear, incrassate or not, not or weakly porose, smooth; axillary hairs numerous (up to or more than ten), strictly axillary and inserted in the central part, with three to five brownish shortly rectangular apical cells and one quadrate hyaline basal cell; archegonia well developed, numerous (up to or more than 10), with numerous paraphyses of eight to > 20 thin-walled or incrassate hyaline or yellowish cells.

Sporophytes not observed.

The new species has been found in the mountains of Madeira (Pico Ruivo, Pico do Areeiro and Lajeado, at or above 1400 m elevation) in wet or moist habitats as slopes or rock crevices in forests dominated by *Erica*

arborea L. and *E. platycodon* subsp. *maderincola* (D.C.McClint.) Rivas Mart *et al*. It grows in association with *Frullania tamarisci* (L.) Dumort., *F. teneriffae* (F.Weber) Nees, *Marsupella emarginata* (Ehrh.) Dumort., *Porella canariensis* (F.Weber) Underw., *Hypnum cupressiforme* Hedw., *H. uncinulatum* Jur., *Isothecium prolixum*, *Ptychomitrium polyphyllum* (Sw.) Bruch & Schimp., and *Racomitrium heterostichum* (Hedw.) Brid.

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APPENDIX 1

Selection of specimens

The bibliographic reference is indicated for sequences included in the molecular analyses that had been previously published; herbarium voucher is indicated for those included in the morphological analyses or obtained in the present study for the molecular analyses. These newly generated sequences are marked with an asterisk. For several samples previous information was available only for part of the regions. In such cases the regions lacking were sequenced from the earlier DNA extractions to ensure that it belonged to the same sample, with three exceptions marked with # where DNA was not available. Information about geographical origin is included only for samples sequenced during the course of this study; ecological features are included only for the ingroup taxa used in the molecular analyses. GenBank accession numbers correspond to [ITS/*trnG*/*trnL-trnF*].

Alleniella complanata. Draper *et al*., 2011 [HQ380945/HQ381000/HQ381076].

Camptochaete arbuscula, Huttunen *et al*., 2012 [FM161087/–/AY306768].

Isothecium algarvicum, Spain, Sierra Grazalema (ESP IP1), Saxicolous, 1250 m, MUB-28584 [HQ380897*/HQ380953*/HQ381007*]; Spain, Sierra Bermeja (ESP IP2), Saxicolous, 500 m, MUB-28583 [HQ380902*/HQ380958*/HQ381012*]; Spain, Sierra Bermeja (ESP IP3), Root, 500 m, MUB-28580 [HQ380899*/HQ380955*/HQ381009*]; Spain, Sierra Bermeja (ESP IP4), Saxicolous, 500 m, MUB-28581 [HQ380900*/HQ380956*/HQ381010*]; Spain, Sierra Bermeja (ESP IP5), Terricolous, 500 m, MUB-28582 [HQ380901*/HQ380957*/HQ381011*]; Spain, Canary Islands, Gran Canaria (ESP GC), Epiphyte, 1120 m, MUB-28579 [HQ380903*/–/HQ381013*]; Spain, Canary Islands, La Gomera (ESP GO1), Saxicolous, 880 m, TFC-Bry-15261/MUB-28578 [HQ380904*/HQ380959*/HQ381014*]; Spain, Canary Islands, La Gomera (ESP GO2), Saxicolous, humid, 790 m, TFC-Bry-12165 [HQ380905*/HQ380960*/HQ381015*]; Spain, Canary Islands, La Palma (ESP PA1), Saxicolous, 1000 m, LG-PALM-1457 [HQ380906*/HQ380961*/HQ381016*]; Spain, Canary Islands, La Palma (ESP PA2), Saxicolous, 1000 m, LG-1574 [HQ380907*/HQ380962*/–]; Spain, Canary Islands, La Palma (ESP PA3), Epiphyte, 1350 m, TFC-Bry-17025/MUB-28771 [HQ380908*/HQ380963*/HQ381017*]; Spain, Canary Islands, El Hierro (ESP HI1), Saxicolous, humid, 1400 m, TFC-Bry-17017/MUB-28774 [HQ380909*/HQ380964*/HQ381018*]; Spain, Canary Islands, El Hierro (ESP HI2), Saxicolous, humid, 1030 m, TFC-Bry-17021/MUB-28775 [HQ380910*/HQ380965*/HQ381019*]; Portugal, Madeira (PRT MD1), Saxicolous, dry, 1800 m, S-B9343 [HQ380911*/HQ380966*/HQ381020*]; Portugal, Madeira (PRT MD2), Saxicolous, 1000 m, S-B9340 [HQ380912*/HQ380967*/HQ381021*]; Portugal, Madeira (PRT MD3), Terricolous, humid, 900 m, S-B9351 [HQ380913*/HQ380968*/HQ381022*]; Portugal, Madeira (PRT MD4), Saxicolous, 900 m, S-B9354 [HQ380914*/HQ380969*/HQ381023*]; Portugal, Madeira (PRT MD5), 900 m, S-B9356 [HQ380915*/HQ380970*/HQ381024*]; Portugal, Madeira (PRT MD6), Epiphyte, 1300 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B9341 [DQ294867/DQ294822/HQ381025*]; Portugal, Madeira (PRT MD7), Saxicolous, 450 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B9347 [DQ294868/DQ294823/HQ381026*]; S (*L. Hedenäs, MA91-91*); S (*L. Hedenäs, MA91-381*); MADJ (*Nóbrega 6154*); MADJ (*Nóbrega 5545*); MADS (*Nóbrega 2162*); MADS (*Nóbrega 2155*); MADJ (*Nóbrega 6155*); MADS (*Nóbrega 2162 'a'*); S (*L. Hedenäs MA91-392*); MADS (*– 2161*); MADS (*Barros 2160*); MADS (*M.de Nóbrega 2458*); MADJ (*Nóbrega 6303*); MADJ (*Nóbrega 6259*); MADJ (*Nóbrega 6494*); MADS (*Nóbrega 2459*); MADS (*Nóbrega 2154*); MADJ (*Nóbrega, Noia 5541*); MADS (*M. de Nóbrega 2153*); MADJ (*Nóbrega 6152*); MADJ (*Nóbrega 5774*); MADS (*M. de Nóbrega 2152*); MADS (*Nóbrega 2156*); S (*L. Hedenäs MA91-287*); S (*L. Hedenäs MA91-290*); S (*S. Fontinha, L. Hedenäs, M. Nóbrega MA91-514*); S (*G.Een, H.Persson*); S (*Nóbrega 261*); MADS (*M. de Nóbrega 2458*); MADJ (*Nóbrega, Paulo 5542*); MADJ (*Nóbrega, Paulo 5543*); MADJ (*Nóbrega 5544*); S (*L. Hedenäs MA91-471*); S (*S. Fontinha, L. Hedenäs MA91-189b*); S (*S. Fontinha, L. Hedenäs MA91-192*); S (*S. Fontinha, L. Hedenäs MA91-198*); S-B9355 (*L. Hedenäs MA91-496*); S (*L. Hedenäs MA91-497*); MADS (*Nóbrega 2157*); MADS (*Nóbrega 2159*); S (*T.J. Bines*); S (*R. Düll X.23*).

Isothecium alopecuroides. Spain, Picos de Europa (ESP IP), 1000 m, Draper *et al*., 2007 [DQ294878/DQ294772/–]; France, Pyrenees (FRA), 1420 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: NMW [DQ294885/DQ294844/HQ381027*]; Georgia, Caucasus (GEO), 2000–2400 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: H [DQ294916/DQ294782/HQ381029*]; Morocco, Rif range (MAR), Saxicolous, 1650 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: MUB-14496 [DQ294869/DQ294771/HQ381030*]; Portugal, Azores, Sao Miguel (PRT AZ), Epiphyte, 200 m, E-00266418 [HQ380916*/HQ380971*/HQ381034*]; Sweden, Västergötland (SWE), Saxicolous, 250 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B91714 [DQ294855/DQ294806/HQ381036*]; Switzerland, Kt. Solothurn (CHE), Saxicolous, 1300 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B94836 [DQ294887/DQ294803/HQ381037*]; S-B105052 (*L. Hedenäs*); S (*J. Greve*); S-B91716 (*L. Hedenäs*); S-B105053

(*L. Hedenäs*); S-B94224 (*L. Hedenäs*); S-B123632 (*L. Hedenäs*).

Isothecium cardotii. Canada, British Columbia (CAN), DUKE-0018250 [HQ380917*/HQ380972*/HQ381038*]; S-B3220 (lectotype); S-B139501; S-B139351.

Isothecium cristatum. Canada, British Columbia (CAN), ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B165251 [DQ294919/DQ294824/HQ381039*]; United States, California (USA), DUKE-0019425 [HQ380918*/HQ380973*/HQ381040*]; S-B47895 (*W. B. Schofield 107732*); S-B118520 (*W. B. Schofield, F. M. Boas 17234*); S-B118521 (*W. B. Schofield, F. M. Boas 17168*); S-B118523 (*W. B. Schofield, F. M. Boas 17384*); S-B118525 (*J. Macoun 14*).

Isothecium holtii. Draper *et al*., 2007 [DQ294923/DQ294834/–]; S-B116388 (G. A. Holt); S-B116389 (G. A. Holt); S-B116390 (D. A. Jones, P. G. M. Rhodes (Bauer, E.: Musci europaei et americani exsiccati 1782)); S-B116401 (N. Hakelier).

Isothecium montanum. Portugal, Madeira (PRT MD1), Saxicolous, 1700 m, Stech *et al*., 2008 [EU477596/–/EU434004]; Portugal, Madeira (PRT MD2), 1550 m, Stech 1044/S-B9261 [HQ380942*/HQ380997*/HQ381070*]; Portugal, Madeira (PRT MD3), Saxicolous, 1700 m, LISU-253210 [KF648803*/KF648833*/KF648818*]; Portugal, Madeira (PRT MD4), Saxicolous, 1700 m, LISU-253209 [KF648804*/KF648834*/KF648819*]; Portugal, Madeira (PRT MD5), Saxicolous, 1500 m, LISU-253212 [KF648802*/KF648832*/KF648817*]; S-B173706 (*G.M. Dirkse, H.M.H. van Melick 17*); S-B9263 (*H. Persson*); S-B9264 (*H. Persson*); S-B43271 (*H. Persson*); LISU-236670 (holotype).

Isothecium myosuroides. Canada, Nova Scotia (CAN), Roots, 35 m, ITS: DUKE-0019487#; *trnG*; *trnL-trnF*: Ryall *et al*., 2005 [HQ380919*/AY747054/AY747014]; Spain, Picos de Europa (ESP IP), Epiphyte, 680 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B183068 [DQ294922/DQ294821/HQ381042*]; Spain, Canary Islands, La Gomera (ESP GO1), Epiphyte, 840 m, TFC-Bry-15238/MUB-28585 [HQ380920*/HQ380974*/HQ381043*]; Spain, Canary Islands, La Gomera (ESP GO2), Epiphyte, 1160 m, TFC-Bry-15235/MUB-28587 [HQ380921*/HQ380975*/HQ381044*]; Spain, Canary Islands, La Gomera (ESP GO3), Epiphyte, 980 m, TFC-Bry-15244/MUB-28589 [HQ380922*/HQ380976*/HQ381045*]; Spain, Canary Islands, Tenerife (ESP TE1), Terricolous, 750 m, TFC-Bry-15259/MUB-28586 [HQ380923*/HQ380977*/HQ381046*]; Spain, Canary Islands, Tenerife (ESP TE2), Terricolous, 790 m, TFC-Bry-15254/MUB-28588 [HQ380924*/HQ380978*/HQ381047*]; Morocco, Rif range (MAR), Epiphyte, 1275 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: MAUAM-Bryo-4396 [DQ294927/DQ294830/HQ381048*]; Portugal, Azores, Flores (PRT AZ), Saxicolous, 700–750 m, S-B42776 [HQ380925*/HQ380979*/HQ381050*]; Portugal, Madeira (PRT MD), Saxicolous, 1250–1350 m, S-B9337 [HQ380926*/HQ380980*/HQ381051*]; United Kingdom, England (GBR), Epiphyte, 35 m, E-00266447 [HQ380927*/HQ380981*/HQ381052*]; S (*S. Medelius*); S (*H. Thedenius*); S (*K. Löfvander*); S (*L. Hedenäs*); S (*L. Hedenäs MA91-382*); S (*H. Persson*); S (*H. Persson*); S (*J. Bornmüller*, *Plantae exs. Canariens* 215, isotyope *I. bornmuelleri*); S-B81113 (*G. Een*); S-B113596 (*L. Hedenäs*); S-B113597 (*L. Hedenäs*); S-B121995 (*N. Hakelier*).

Isothecium myosuroides* var. *brachythecioides. Ireland, W. Galway (IRL), Epiphyte, 44 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B97668 [DQ294921/DQ294835/HQ381053*]; United Kingdom, Scotland (GBR1), Saxicolous, humid, 195 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B97667 [DQ294926/DQ294836/HQ381054*]; United Kingdom, Scotland (GBR2), Saxicolous, 5 m, E-00266448 [HQ380928*/HQ380982*/HQ381055*]; Norway, Hordaland District (NOR), Saxicolous, 25 m, ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: E-00197979 [DQ294925/DQ294838/HQ381056*]; S-B118336 (*N. Hakelier*); S-B118347 (*P. A. H. Arnell 575*); S-B118348 (*P. A. H. Arnell 293*); S-B74090 (*G. Een UK523*).

Isothecium prolixum. Portugal, Azores, Terceira (PRT AZ1), Saxicolous, ITS; *trnL-trnF*: Stech *et al*., 2008; *trnG*: LISU-RG-011204/2# [EU477598/HQ380983*/EU434006]; Portugal, Azores, Terceira (PRT AZ2), Epiphyte, 660 m, Stech 1045/S-B42630 [HQ380929*/HQ380984*/HQ381057*]; Portugal, Azores, Flores (PRT AZ3), Epiphyte, 780 m, Stech 1046/S-B42628 [HQ380930*/HQ380985*/HQ381058*]; Portugal, Azores, Flores (PRT AZ4), Saxicolous, dry, 725 m, Stech 1047/S-B42631 [HQ380931*/HQ380986*/HQ381059*]; Portugal, Azores, São Jorge (PRT AZ5), L-Stech 08–466 [HQ380932*/HQ380987*/HQ381060*]; Portugal, Azores, São Jorge (PRT AZ6), L-Stech 08–486 [HQ380933*/HQ380988*/HQ381061*]; Portugal, Madeira (PRT MD1), Saxicolous, 860 m, MUB-15632 [HQ380934*/HQ380989*/HQ381062*]; Portugal, Madeira (PRT MD2), Saxicolous, 1000 m, L-Stech 04–033 [HQ380935*/HQ380990*/HQ381063*]; Portugal, Madeira (PRT MD3), Epiphyte, 1000 m, L-Stech 05–144 [HQ380936*/HQ380991*/HQ381064*]; Portugal, Madeira (PRT MD4), Saxicolous, 500 m, L-Stech 04–156 [HQ380937*/HQ380992*/HQ381065*]; Portugal, Madeira (PRT MD5), Terricolous, 1300 m, L-Stech 04–531 [HQ380938*/–/HQ381066*]; Portugal, Madeira (PRT MD6), Epiphyte, 1450 m, L-Stech 04–231 [HQ380939*/HQ380993*/HQ381067*]; Portugal, Madeira (PRT MD7), Epiphyte, 880 m, L-Stech 04–450 [HQ380940*/HQ380994*/HQ381068*]; Portu-

gal, Madeira (PRT MD8), Saxicolous, 800 m, L-Stech 04–306 [HQ380941*/HQ380995*/HQ381069*]; Portugal, Madeira (PRT MD9), Epiphyte, 1050 m, ITS; *trnL-trnF*: Stech *et al*., 2008; *trnG*: LISU-04–192# [EU477597/HQ380996*/EU434005]; Portugal, Madeira (PRT MD10), Epiphyte, 1200 m, LISU-253218 [KF648790*/KF648820*/KF648805*]; Portugal, Madeira (PRT MD11), Epiphyte, 1000 m, LISU-253220 [KF648791*/KF648821*/KF648806*]; Portugal, Madeira (PRT MD12), Terricolous, 1075 m, LISU-253225 [KF648792*/KF648822*/KF648807*]; Portugal, Madeira (PRT MD13), Terricolous, 1100 m, LISU-253224 [KF648793*/KF648823*/KF648808*]; Portugal, Madeira (PRT MD14), Epiphyte, 850 m, LISU-253219 [KF648794*/KF648824*/KF648809*]; Portugal, Madeira (PRT MD15), 1005 m, LISU-253216 [KF648795*/KF648825*/KF648810*]; Portugal, Madeira (PRT MD16), 955 m, LISU-253214 [KF648796*/KF648826*/KF648811*]; Portugal, Madeira (PRT MD17), 1205 m, LISU-253221 [KF648797*/KF648827*/KF648812*]; Portugal, Madeira (PRT MD18), 1175 m, LISU-253213 [KF648798*/KF648828*/KF648813*]; Portugal, Madeira (PRT MD19), 910 m, LISU-253222 [KF648799*/KF648829*/KF648814*]; Portugal, Madeira (PRT MD20), 1115 m, LISU-253215 [KF648800*/KF648830*/KF648815*]; Portugal, Madeira (PRT MD21), Saxicolous, 1700 m, LISU-253211 [KF648801*/KF648831*/KF648816*]; S (*Costa*); S (*L. Hedenäs MA90-255*); S (*L. Hedenäs MA90-256*); S (*L. Hedenäs MA91-344*); S (*M. Nóbrega, H. Persson*); S (*L. Hedenäs MA91-384*); S (*C.H.C. Pickering*); S (*H. Persson*); S (*G. Een, H. Persson*); S (*G. Een, H. Persson*); S (*H. Persson*); S-B9225 (*L. Hedenäs MA90-247*); S (*L. Hedenäs MA90-93*); S (*L. Hedenäs MA90-105*); S (*L. Hedenäs MA90-107*); S (*L. Hedenäs MA90-112*); S (*L. Hedenäs MA90-132*); S-B9231 (*L. Hedenäs MA90-200*); S (*L. Hedenäs MA90-210*); S (*L. Hedenäs MA90-224*); S (*L. Hedenäs MA90-231*); S (*L. Hedenäs MA90-76*); S (*H. Persson*); S (*H. Persson*); S (*G. Een, H. Persson*); S (*M. Nóbrega, H. Persson*); S (*T. & C. Friedländer*); S (*Nóbrega*); S (*M. Nóbrega*); S (*H. Persson*); S (*Nóbrega*); S (*Mandon 33*); S (*Mandon*); S (*Nóbrega*); S (*G. Een, H. Persson*); MADS (*Nóbrega 1626*, as *Homalothecium barbelloides*); S (*H. Persson*); S (*H. Persson*); S (*H. Persson*); S (*H. Persson*); S (*L. Hedenäs MA91-288*); S (*L. Hedenäs MA91-133*); S (*G. Een, H. Persson*); S (*G. Een, H. Persson*); S (*G. Een, H. Persson*); S (*L. Hedenäs MA91-124*); S (*Düll*); S (*Nóbrega*); S (–); S (*H. Persson*); S (*G. Een, H. Persson*); S (*H. Persson*); S (*L. Hedenäs MA91-465*); S (*C.H.C. Pickering*); S (Ex. herb *S.O.Lindberg*); S (*R. Fritze*); S (*Kny*); S (*R. Fritze* (?)); S (*R. Fritze*); S (*L. Hedenäs MA91-8*); S (*L. Hedenäs MA91-26*); S (*L. Hedenäs MA91-494*); S (*L. Hedenäs MA91-76*); S (*L. Hedenäs MA91-142*); S (*H. Persson*); S (*L. Hedenäs MA91-131*); S (*H. Persson*); S (*H. Persson*).

Isothecium stoloniferum. Canada, Winchelsea Island (CAN), ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B165344 [DQ294920/DQ294826/HQ381071*].

Isothecium subdiversiforme. Japan, Honshu (JPN), ITS; *trnG*: Draper *et al*., 2007; *trnL-trnF*: S-B117361 [DQ294918/DQ294827/HQ381073*]; S-B117362 (*M. Mizutani 15153*); S-B117363 (*M. Mizutani 9246*); S-B117364 (*S. Okamura*); S-B117365 (*R. Toyama*).

Lembophyllum divulsum. Huttunen *et al*., 2012 [FM161146/–/AY306769].

Leptodon smithii. Draper *et al*., 2011 [HQ380943/HQ380998/HQ381074].

Neckera cephalonica. Draper *et al*., 2011 [HQ380944/HQ380999/HQ381075].

Neckera pumila. Draper *et al*., 2011 [HQ380947/HQ381002/HQ381078].

Nogopterium gracile. ITS; *trnL-trnF*: Huttunen *et al*., 2012; *trnG*: Stech *et al*., 2011 [HE660012/HQ268381/HE717062].

Rigodium implexum. Huttunen *et al*., 2012 [FM161209/–/AF543547].

Weymouthia mollis. Huttunen *et al*., 2012 [FM161237/–/AY306847].

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Consensus phylogram for the ingroup taxa (*Isothecium* genus) based on Bayesian inference resulting from the analysis of the two genomes analysed, including a simple coded indel matrix. A: nuclear ITS; B: chloroplast *trnG* and *trnL-trnF* data sets combined. Numbers above the branches indicate posterior probability node support. Values shown are those above 0.90. Abbreviations for the location data correspond to Spain (ESP), Iberian Peninsula (IP), La Palma Island (PA), El Hierro Island (HI), La Gomera Island (GO), Gran Canaria Island (GC), Tenerife Island (TE), Portugal (PRT), Madeira Island (MD), Azores (AZ), Switzerland (CHE), Morocco (MAR), Sweden (SWE), France (FRA), Georgia (GEO), Canada (CAN), United States (USA), Ireland (IRL), Norway (NOR), and Great Britain (GBR), numbers correspond to the samples listed in the Appendix.