









## Research Article

## Seeking the identity of an enigmatic moss by embracing phylogenomics

Pablo Aguado-Ramsay<sup>1,2</sup> , Tamara Villaverde<sup>3</sup> , Ricardo Garilleti<sup>4</sup> , J. Gordon Burleigh<sup>5</sup> , Stuart F. McDaniel<sup>5</sup> , Maren Flagmeier<sup>1</sup> , Jorgen Nieuwkoop<sup>6</sup>, Arno van der Pluijm<sup>7</sup>, Florian Hans<sup>8</sup>, Francisco Lara<sup>1,9†\*</sup> , and Isabel Draper<sup>1,9†</sup> 

<sup>1</sup>Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, C. Darwin, 2, Madrid 28049, Spain

<sup>2</sup>Doctoral Program in Biology, Doctoral School (EDUAM), Universidad Autónoma de Madrid, C. Francisco Tomás y Valiente, 2, Madrid 28049, Spain

<sup>3</sup>Departamento de Biología, Geología, Física y Química Inorgánica, Universidad Rey Juan Carlos, C. Tulipán s/n, Móstoles 28933, Spain

<sup>4</sup>Departamento de Botánica y Geología, Facultad de Farmacia, Universidad de Valencia, Avda. Vicente Andrés Estelles s/n, Burjassot 46100, Spain

<sup>5</sup>Department of Biology, University of Florida, 220 Bartram Hall, Gainesville, FL 32611, Florida, USA

<sup>6</sup>Vluchtheuvelstraat 6, Dreumel, BK 6621, The Netherlands

<sup>7</sup>Visserskade 10, Hank, GL 4273, The Netherlands

<sup>8</sup>Kreuzweiler Straße 22, Perl-Sinz D-66706, Germany

<sup>9</sup>Centro de Investigación en Biodiversidad y Cambio Global, Universidad Autónoma de Madrid, C. Darwin, 2, Madrid 28049, Spain

†These authors are joint senior authors on this work.

\*Author for correspondence. E-mail: francisco.lara@uam.es

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**Abstract** Currently, a wide range of genomic techniques is available at a relatively affordable price. However, not all of them have been equally explored in bryophyte systematics. In the present study, we apply next-generation sequencing to identify samples that cannot be assigned to a taxon by morphological analysis or by Sanger sequencing methods. These samples correspond to a moss with an enigmatic morphology that has been found throughout Western Europe over the last two decades. They exhibit several anomalies in the gametophyte and, on the rare occasions that they appear, also in the sporophyte. The most significant alterations are related to the shape of the leaves. Morphologically, all specimens correspond to mosses of the genus *Lewinskya*, and the least modified samples are potentially attributable to the *Lewinskya affinis* complex. Specimen identifications were first attempted using up to seven molecular markers with no satisfactory results. Thus, we employed data generated from targeted enrichment using the GoFlag 408 flagellate land plant probe set to elucidate their identity. Our results demonstrate that all the enigmatic samples correspond to a single species, *L. affinis* s.str. This approach provided the necessary resolution to confidently identify these challenging samples and may be a powerful tool for similar cases, especially in bryophytes.

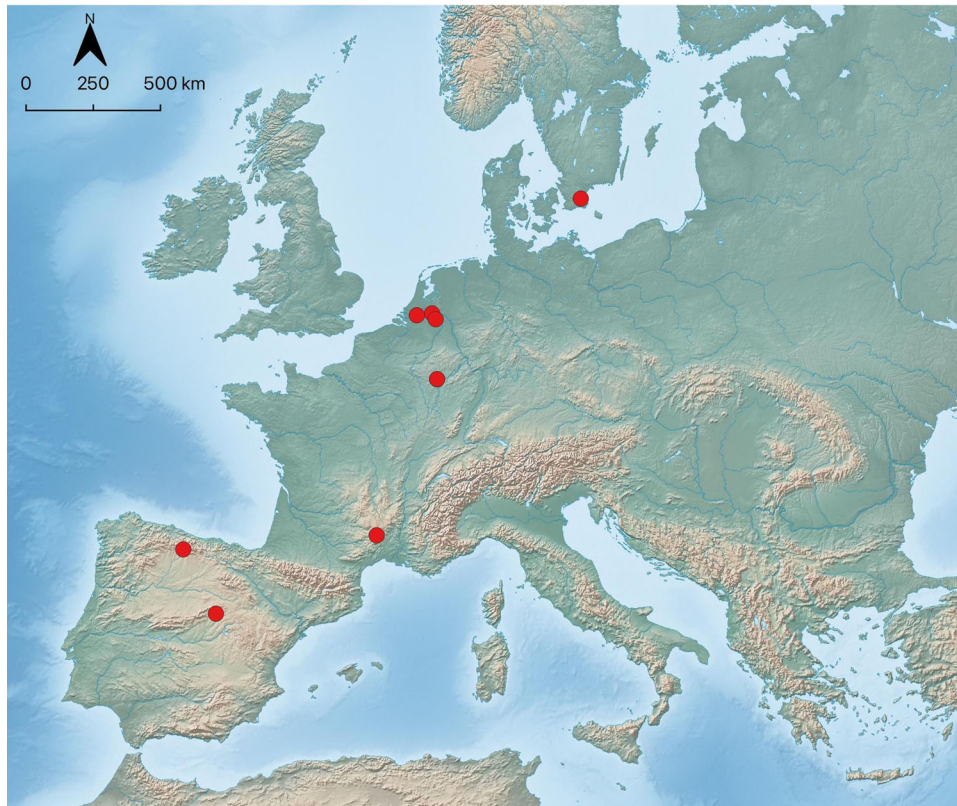
**Key words:** Bryophyta, GoFlag 408, *Lewinskya affinis*, Orthotrichaceae, targeted enrichment, taxonomy.

## 1 Introduction

A major goal of systematics is species delimitation (Sites & Marshall, 2003; Wiens, 2007; Stanton et al., 2019), which can be particularly difficult for small and phenotypically inconspicuous organisms (Riddle et al., 2011; Hortal et al., 2015), such as mosses (Bryophyta Schimp.; Medina et al., 2011). These nonvascular plants, comprising an estimated 12 000–13 000 species (Frey & Stech, 2009; Brinda & Atwood, 2023), represent the most diverse group of bryophytes (Bryobiotina Trevis.). Within mosses, Orthotri-

chaceae Arn. is the second most diverse family (Frey & Stech, 2009; Brinda & Atwood, 2023), with nearly 900 described taxa (Goffinet & Vitt, 1998; Goffinet et al., 2004). Molecular data have enabled important advances in elucidating the evolutionary framework of the subfamily Orthotrichoideae Broth. (e.g., Draper et al., 2021, 2022; Aguado-Ramsay et al., 2022), unraveling a much greater diversity than previously thought with the description of numerous new species (e.g., Garilleti et al., 2015; Eckstein et al., 2018; Lara et al., 2018, 2020; Vigalondo et al., 2019, 2020; Draper et al., 2022).

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**Fig. 1.** Localities of origin of the anomalous moss samples.

Several long-term large-scale inventories of bryophytes throughout Europe conducted in the past decades revealed several moss specimens with enigmatic morphology (Fig. 1). These specimens have leaves with anomalies in shape and structure; different types of leaf apices (acute, flagelliform, obtuse, and rounded), margins (flat, and variably recurved to revolute), and even costae (well differentiated, incomplete, or absent) can be found in the same individual. The overall shape of the leaves can vary considerably, from lanceolate to oblong-lanceolate and also lanceolate-ligulate, ovate-lanceolate, or spatulate. In addition, the rhizoids tend to climb up the stem, and the calyptrae are mostly glabrous, smooth, and sometimes falciform. Other gametophytic characteristics, especially from non or scarcely modified leaves when present, place these peculiar specimens within the family Orthotrichaceae, and singularities suggest the ascription of these specimens to *Lewinskya* F. Lara, Garilleti & Goffinet (as outlined in Lara et al., 2016), a worldwide spread genus with more than 70 taxa, 20 of which are found in Europe and Macaronesia (Hodgetts et al., 2020; Vigalondo et al., 2020; Kiebach et al., 2022).

The first of these anomalous moss samples had highly modified gametophytes and came from the Netherlands and Luxembourg. These specimens remained unassigned to any taxon after a careful revision by several experienced bryologists. Eventually, additional samples were found in various parts of Western Europe, from Spain to southern Sweden, and some of these had unaltered leaves. Specimens with sporophytes, although not entirely uniform, allowed its

ascription to the *Lewinskya affinis* complex, as defined by Vigalondo et al. (2019, 2020). Considering the recent recognition of the diversity of this group, and that the abnormalities concern taxonomically important characters (Lewinsky, 1993), one of the starting hypotheses was that the samples could represent an undescribed species with high morphological variation. An alternative hypothesis is that this variation could represent malformations of individuals corresponding to one or several existing species. Morphological characters and scarce material prevent us from rejecting either hypothesis.

Consequently, in this study, we use molecular data to help resolve the taxonomic placement of the anomalous moss specimens. Unlike morphological observations, molecular data are not influenced by the environmental conditions in which the samples are grown and can help to determine whether the observed differential characters, or other previously overlooked characters, are of taxonomic importance (e.g., Steele & Pires, 2011; Medina et al., 2012). An initial phylogenetic analysis using seven molecular markers obtained by Sanger sequencing yielded many unresolved evolutionary relationships among the analyzed specimens (results not shown). To overcome this lack of resolution, we used phylogenomic techniques capable of generating data from hundreds of loci. Specifically, we used a targeted enrichment approach with the GoFlag 408 flagellate land plant probe set (Breinholt et al., 2021), which targets 408 exons found in 229 single or low-copy nuclear genes. GoFlag 408 has already generated genome-scale data sets that have

resolved relationships among vascular (Fawcett et al., 2021; Mendez-Reneau et al., 2023; Vieira Lima et al., 2023) and nonvascular plants (Budke et al., 2022; Bechteler et al., 2023; Jauregui-Lazo et al., 2023), including Orthotrichoideae (Draper et al., 2022). However, it has not been used for species delimitation or for identifying particularly challenging or enigmatic samples. Here, we investigate whether the anomalous specimens correspond to one or more species of the *L. affinis* complex that have undergone teratological processes using genome data. Moreover, we were also investigating whether the genomic data from the GoFlag probe set provided the necessary resolution to identify challenging samples.

## 2 Material and Methods

### 2.1 Morphological evaluation

We evaluated nearly 90 morphological characters that are useful in the family (see Vigalondo et al., 2016). Microscopic preparations of leaves, caulidia, and rhizoids, as well as the sporophyte when present, were made for all samples (Table 1).

### 2.2 Taxon sampling

We sampled five specimens of the anomalous moss (NLD-2, NLD-3, LUX-1, SWE-1, and ESP-2) and 12 other species of *Lewinskya* for the phylogenetic analysis. Our sampling focused on the *Lewinskya affinis* complex (Vigalondo et al., 2019, 2020) and morphologically similar species. One of the specimens corresponding to *L. affinis* s.str. (Brid.) F. Lara, Garilleti & Goffinet were obtained from a sample that also contained one anomalous moss (specimen NLD-3). To prevent contamination, only the top of a single gametophyte shoot was selected for DNA extraction. If the shoots of a specimen were too small, we selected two adjacent ones (with sporophytes if possible). We preserved the rest of the gametophyte, together with the sporophyte when present, on a microscope slide fixed with Kaiser's glycerol gelatine to allow morphological re-evaluation. For the phylogenetic analyses, we also included 30 sequences from Draper et al. (2022) that were obtained using the same procedure. Thus,

the final sampling includes 26 taxa of *Lewinskya* (ca. 40% of the accepted species), nine samples of all genera currently accepted in subtribe Lewinskyinae F. Lara, Garilleti & Draper (*Atlantichella* F. Lara, Garilleti & Draper, *Plenogemma* Plášek, Sawicki & Ochrya, *Pulviger* Plášek, Sawicki & Ochrya, *Rehubryum* F. Lara, Garilleti & Draper and *Ulota* D. Mohr), and five representatives from *Orthotrichum* Hedw. (subtribe Orthotrichinae F. Lara, Garilleti & Draper) and *Zygodon rupestris* Schimp. ex Lorentz (subtribe Zygodontae Engler) as outgroups. Voucher information for all 47 samples is available in Table S1.

### 2.3 DNA isolation, target enrichment, sequencing, and assembly

DNA was extracted using a modified CTAB protocol, as described in Breinholt et al. (2021). The library construction, targeted enrichment, and sequencing were performed by RAPiD Genomics (Gainesville, FL, USA), following the protocol in Breinholt et al. (2021). The raw Illumina sequence reads for each target locus were assembled and aligned using the iterative baited assembly (IBA) pipeline as in Breinholt et al. (2021) and Draper et al. (2022). Two different data sets were generated: (i) the “full matrix,” which includes all 47 samples, and (ii) the “reduced matrix,” which includes 13 samples from a reduced set of species to which the anomalous samples proved to be more closely related. The IBA pipeline seeks to extend the assemblies beyond the target regions, which are relatively conserved exons. For the “full matrix” analyses, we used locus alignments containing only the target regions, but for the “reduced matrix” analyses, we used locus alignments that included both the exonic target regions and the mostly noncoding flanking regions. All locus alignments were pruned using Gblocks (Castresana, 2000; Talavera & Castresana, 2007) with varying degrees of stringency (-b1=20 -b2=20 -b3=8 -b4=8 -b5=h for the “full matrix,” and -t=d -b1=10 -b2=10 -b3=8 -b4=8 -b5=h for the “reduced matrix”). Summary statistics were calculated using AMAS (Borowiec, 2016), and matrices were visualized with Geneious Prime 2022.2.2 (<http://www.geneious.com/>). To minimize missing data, loci with <20 terminals were removed for the “full matrix” (except for L54 and L117, which contain several

**Table 1** Collection data of all known samples with individuals showing anomalous morphology

ID	Country	Collectors	Collection date	Voucher
NLD-1	The Netherlands, Aert-Eloyenbosch	A. Boesveld	2001-03-20	MAUAM 5367 (dupl. ex. herb. A.v.d Pluijm 2311)
NLD-2	The Netherlands, Molenheide	R. v. d. Bosch	2001-10-12	MAUAM 5368 (dupl. ex. herb. A.v.d Pluijm 2415)
LUX-1	Luxembourg, Kehlen	F. Hans	1994-12-14	MAUAM 5247
LUX-2	Luxembourg, Kehlen	F. Hans	1994-12-14	MAUAM 5370
ESP-1	Spain, León, Crémenes	Draper & Medina	2004-05-22	MAUAM 5244
SWE-1	Sweden, Kristinehov	F. Lara with T. Hallingbäck & N. Lönnell, 1707/50	2017-07-21	MAUAM 5246
NLD-3	The Netherlands, Altforst	J. Nieuwkoop	2019-06-01	MAUAM 5369 (dupl. ex. herb. J. Nieuwkoop 2019197)
FRA-1	France, Florac, La Grange	J. Nieuwkoop	1989-10-01	J. Nieuwkoop 89669
ESP-2	Spain, Guadalajara	F. Lara 2012/01	2020-12-04	MAUAM 5245

Samples are arranged in the order in which they were received or found, and thus studied.

species of the ingroup), along with L35, L83, and L263, for which the alignment appeared anomalous. For the “reduced matrix,” we removed loci with <10 terminals.

## 2.4 Phylogenetic inference

We used two approaches for the phylogenetic inference: (i) a total evidence analysis using maximum likelihood (ML) for the concatenated supermatrix and (ii) a species tree approach that accounts for the multiple species coalescent (MSC).

For the total evidence analysis, individual loci for the “full matrix” were concatenated into a supermatrix using AMAS. We performed an ML phylogenetic analysis using IQ-TREE 2.2.3 (Nguyen et al., 2015; Minh et al., 2020), with an automatic model selection using ModelFinder (“TEST” option; Kalyaanamoorthy et al., 2017) with the approximate likelihood ratio test (“aLRT” option), 1000 bootstrap replicates and 1000 ultrafast bootstrap replicates (“bb” option; Hoang et al., 2018), and with an additional step to further optimize UFBoot trees by nearest neighbor interchange (“bnni” option). We analyzed the “reduced matrix” with the same options, but with partition models (Chernomor et al., 2016) “-Q”, “TESTMERGE” for ModelTest, and “-sampling GENESITE.” The resulting phylogenetic trees were visualized and edited by using TreeGraph2 (Stöver & Müller, 2010).

For the MSC analysis, we first constructed gene trees with RAXML-NG 1.1 (Kozlov et al., 2019), doing both an ML search and bootstrapping (“-all” option) using the GTR + G model. Nodes in the resulting gene trees with  $\leq 10\%$  bootstrap support (BS) were collapsed using Newick Utilities (Junier & Zdobnov, 2010) as suggested by Zhang et al. (2018) and Mirarab (2023). We then inferred the species tree from the collapsed gene trees using ASTRAL III 5.7.1 (Zhang et al., 2018). Quartet support values were calculated with the “-t 2” option. Pie charts with quartet values were plotted using R 4.3.0 (R Core Team, 2023) using the packages ape (Paradis & Schliep, 2019), ggimage, ggtree (Yu et al., 2017), and treeio (Wang et al., 2020). In addition, we calculated the gene concordance factor (gCF) and site concordance factor (sCF) with IQ-TREE 2.2.3.

## 3 Results

### 3.1 Morphological evaluation

The anomalous samples showed great morphological variability (Figs. 2–4; Table S2). Within gametophytes, the morphological anomalies mainly involve characteristics of the leaves. Leaf variability can be grouped into four basic types of syndromes (Fig. 3). Type I corresponds to lanceolate-ligulate leaves with rounded or obtuse apices, revolute margins reaching even the apex in some cases, and with absent or only slightly differentiated costae; this type was observed in samples NLD-2, NLD-3, LUX-1, LUX-2, and partially in NLD-1. Type II corresponds to ovate-lanceolate to spatulate or long linear to strap-shaped leaves with rounded apices, plane margins, and absent or more or less weakly differentiated costae; these leaves are usually contorted or undulated when dry and were observed in SWE-1 and partially in NLD-1. Type III corresponds to lanceolate

leaves with long acuminate to flagelliform apices, margins of variable forms from narrowly recurved to almost totally plane, and with absent or only weakly differentiated costae; this type was observed in samples NLD-2, NLD-3, LUX-1, LUX-2 and partially in NLD-1. Finally, type IV corresponds to ovate-lanceolate to oblong-lanceolate leaves with broadly acute apices, margins recurved to revolute, and weak to almost well-developed costae; this type was observed in ESP-2 and FRA-1. In several samples, a variable proportion of leaves also had no appreciable alterations and were very similar to those of the European species of the *Lewinskya affinis* complex (Vigalondo et al., 2019).

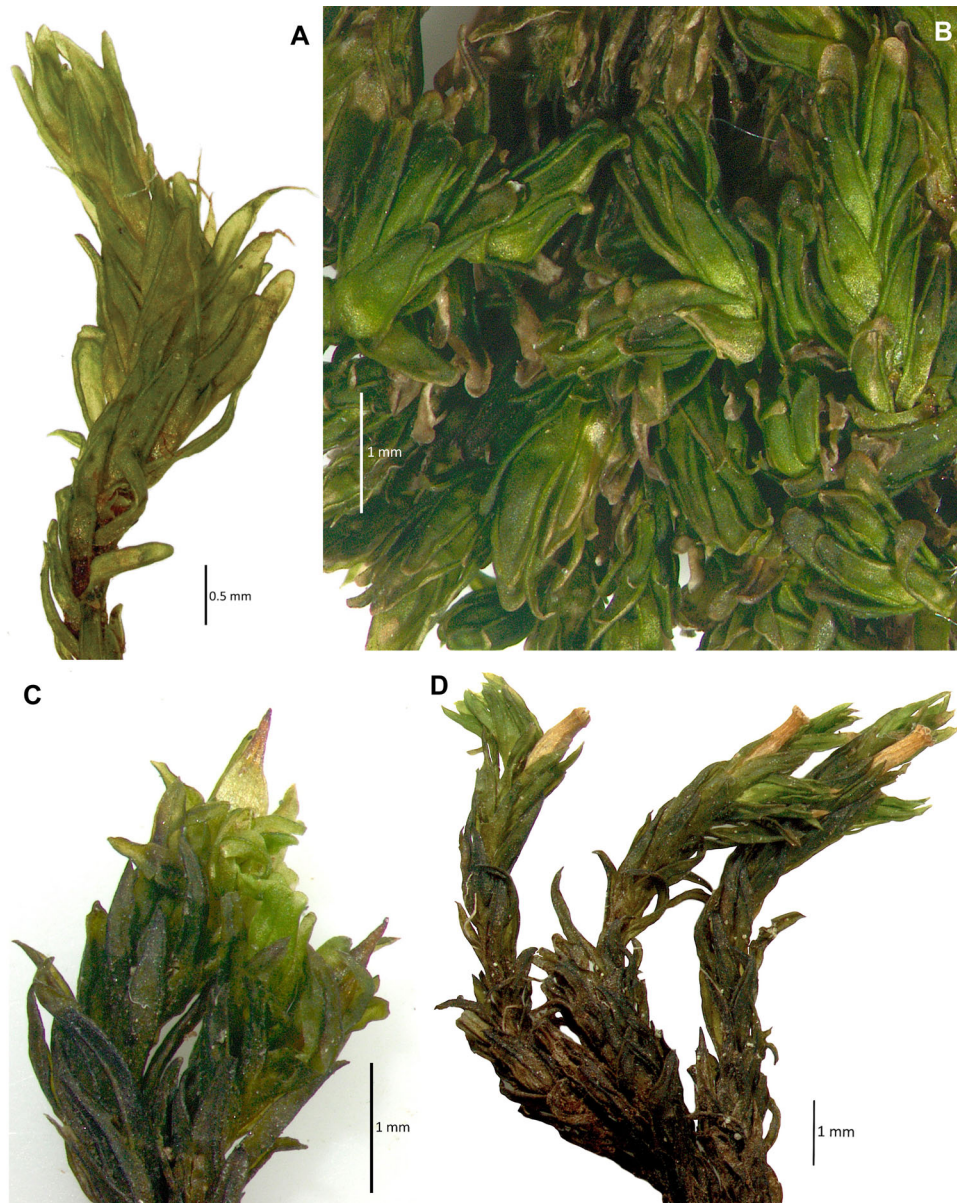
Despite this simplification, the heterogeneity among samples in leaf morphology is enormous (Table S2). There are samples in which all the leaves are anomalous, others where shoots with and without anomalous leaves coexist, and others where only a single shoot has anomalous leaves. Also, multiple types of anomalous leaves may occur in the same sample. Abnormalities can also be found in other gametophyte structures. For instance, the few calyptrae available in the enigmatic samples are mostly glabrous, whereas they are more or less hairy in all other European *Lewinskya* species. Moreover, in sample ESP-2, this structure exhibits a smooth and curved appearance, lacking plicae, which are characteristics inconsistent with those observed in the calyptrae of the Orthotricheae tribe. In addition, rhizoids are not only abundant at the base of the main stems but also ascending along them, particularly at the base of the branches.

Anomalies in the sporophytes were generally less pronounced; however, they equally limit the possibility of confidently identifying the samples. Only a few samples exhibiting anomalous leaves contain sporophytes. The capsules of these sporophytes are similar to *L. affinis* or other species within the complex, although they often display deviations from the typical morphology. For instance, in sample SWE-1, the capsules appear almost exserted, with the urn elongated, very weakly ribbed, and have an operculum with an extraordinarily long rostrum (Fig. 4A). Similarly, the peristome characteristics do not provide a basis for precise identification. In the case of sample ESP-2, where the capsules exhibit a morphology more consistent with that of *L. affinis*, the endostome segments appear smooth, deviating from the characteristics observed in any known species within the complex (Fig. 4B).

### 3.2 Target enrichment success and data quality

The phylogenetic analyses included 47 samples from 42 taxa, 17 of them newly sequenced in this study, and the rest from Draper et al. (2022). We recovered most of the targeted loci from all the newly sequenced samples, including sample LUX-1 collected almost 30 years ago. The “full matrix,” with only the target exon regions from all the samples, included sequences from 385 of the 408 possible loci, with a total alignment length of 68 231 base pairs (bp) and 4.83% missing data. Of these loci, 381 included sequences from at least half of the samples, and 328 (ca. 80%) included sequences from at least 90% of the samples. The “reduced matrix” included 372 loci and had a total length of 196 659 bp, 367 (ca. 90%) of which included at least 90% of the sequenced terminals, resulting in 1.36% of missing data. Summary statistics for the





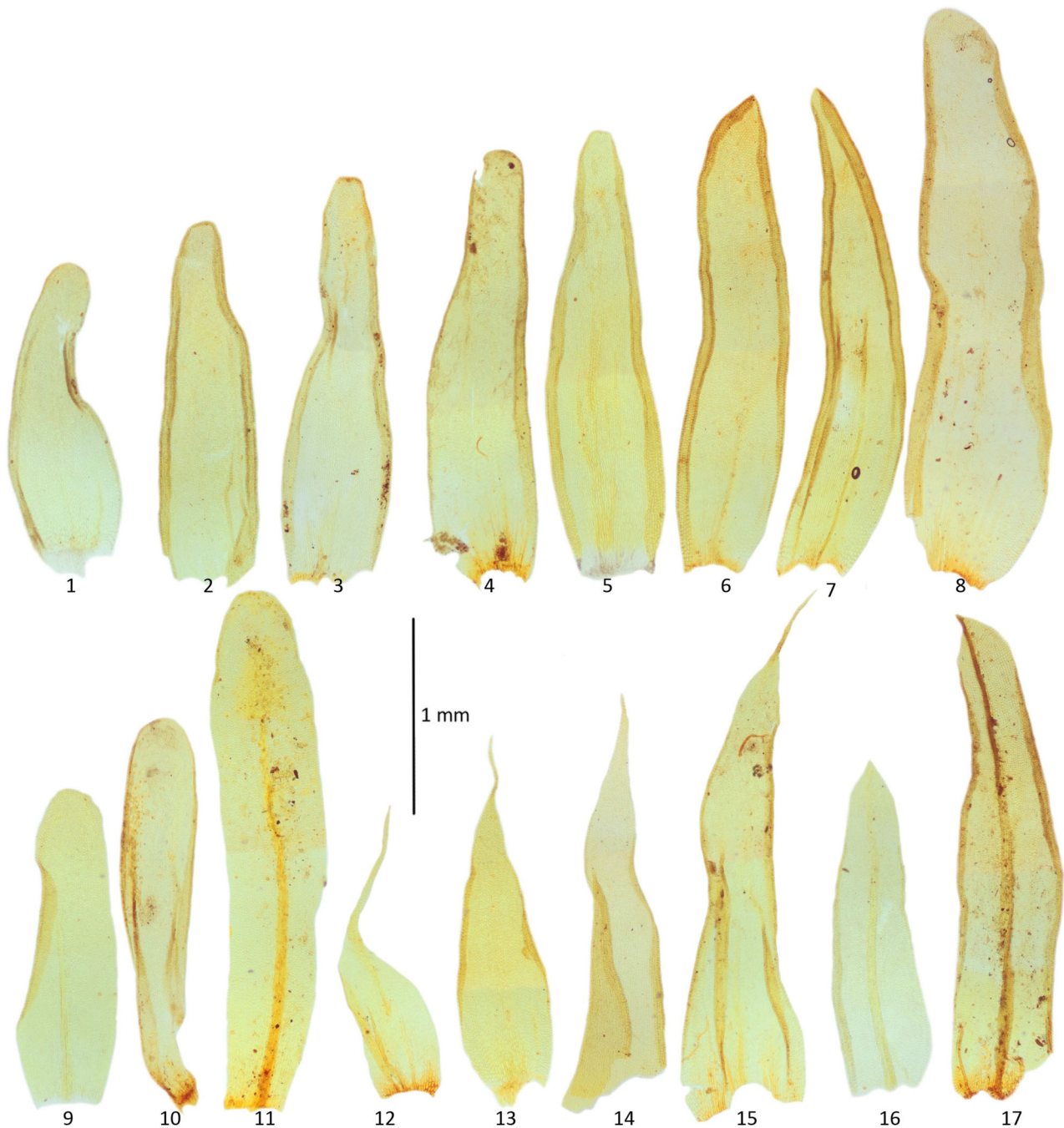
**Fig. 2.** Examples of anomalous plants. **A**, Gametophyte shoot from sample NLD-2 showing all its leaves modified; two types of leaves can be clearly distinguished. **B**, Detail of sample NLD-3 showing several stems covered by anomalous leaves, all of them similar to each other. **C**, Detail of a shoot from sample ESP-2 showing modified leaves and smooth calyptrae. **D**, Tuft from sample ESP-2 in which nonanomalous leaves (i.e., leaves similar to those of species in the *Lewinskya affinis* complex) predominate.

phylogenetic data sets are available in Table S3. The raw sequence reads from the newly sequenced samples are available as fastq files in the NCBI Sequence Read Archive (SRA) under the BioProject number PRJNA973030, and the raw sequence read files from previously sequenced samples are available under BioProject PRJNA819401 (see Draper et al., 2022). Accession numbers of each sample are indicated in Table S1.

### 3.3 Phylogenetic reconstruction

Both ML and MSC phylogenetic analyses of the “full matrix,” inferred from exon regions, produced generally well-resolved

and strongly supported trees (Fig. 5), with mostly low sCF and gCF values (Table S4). Branch support values within *Lewinskya* are high, with the exception of four nodes with <90% BS in ML analysis and three nodes with <0.9 local posterior probabilities (LPP) in the MSC analysis. Within the *L. affinis* complex, clades are maximally supported except for the node that integrates the Old and New World representatives (78 BS/0.87 LPP), which also has low gCF and sCF values (5.65 and 16.8, respectively). The anomalous moss samples are retrieved in a maximally supported lineage with *L. affinis* s.str samples, in a clade with moderate concordance factors (sCF = 27.2 and gCF = 55). However,



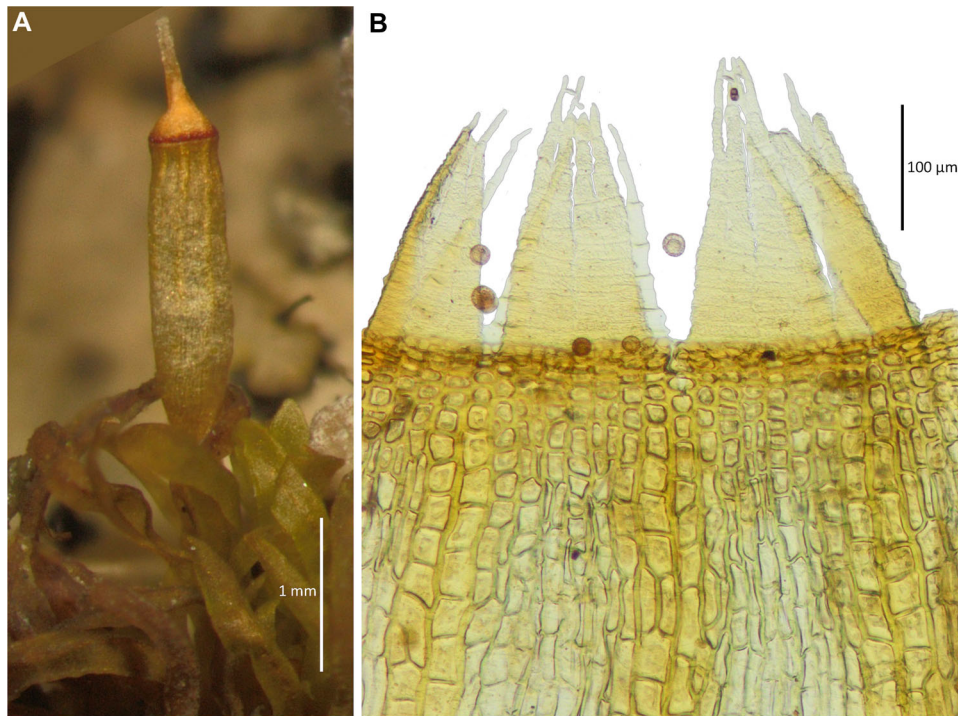
**Fig. 3.** Variability of leaves with anomalies, grouped according to the types described in the text: 1–8: type I; 9–11: type II; 12–15: type III; 16–17: type IV.

branch support values, branch lengths, and concordance factor values are low for the evolutionary relationships among the anomalous samples and *L. affinis*.

Analyses of the “reduced matrix,” which also includes the flanking region of the targeted loci, retrieved higher branch support values and concordance factors (Fig. 6; Table S4). The lineage comprising Old and New World samples within the *L. affinis* complex is here retrieved with a 91% BS in the ML analyses. Nevertheless, gCF and sCF values are still low (15.3 and 29.3, respectively). This is the only node that is not

congruent between the ML and the MSC inferred trees, in which *L. pacifica* Vigalondo, F. Lara & Garilleti, and *Lewinskya pseudoaffinis* Vigalondo, F. Lara & Garilleti appear in an early diverging lineage with maximum support, instead of *Lewinskya arida* Vigalondo, F. Lara & Garilleti. All phylogenetic reconstructions recover the anomalous moss specimens in a maximally supported clade nested with the two *L. affinis* specimens with high gCF and sCF values (75.3 and 97.7, respectively). Evolutionary relationships within this clade are not well supported and have low concordance factors.





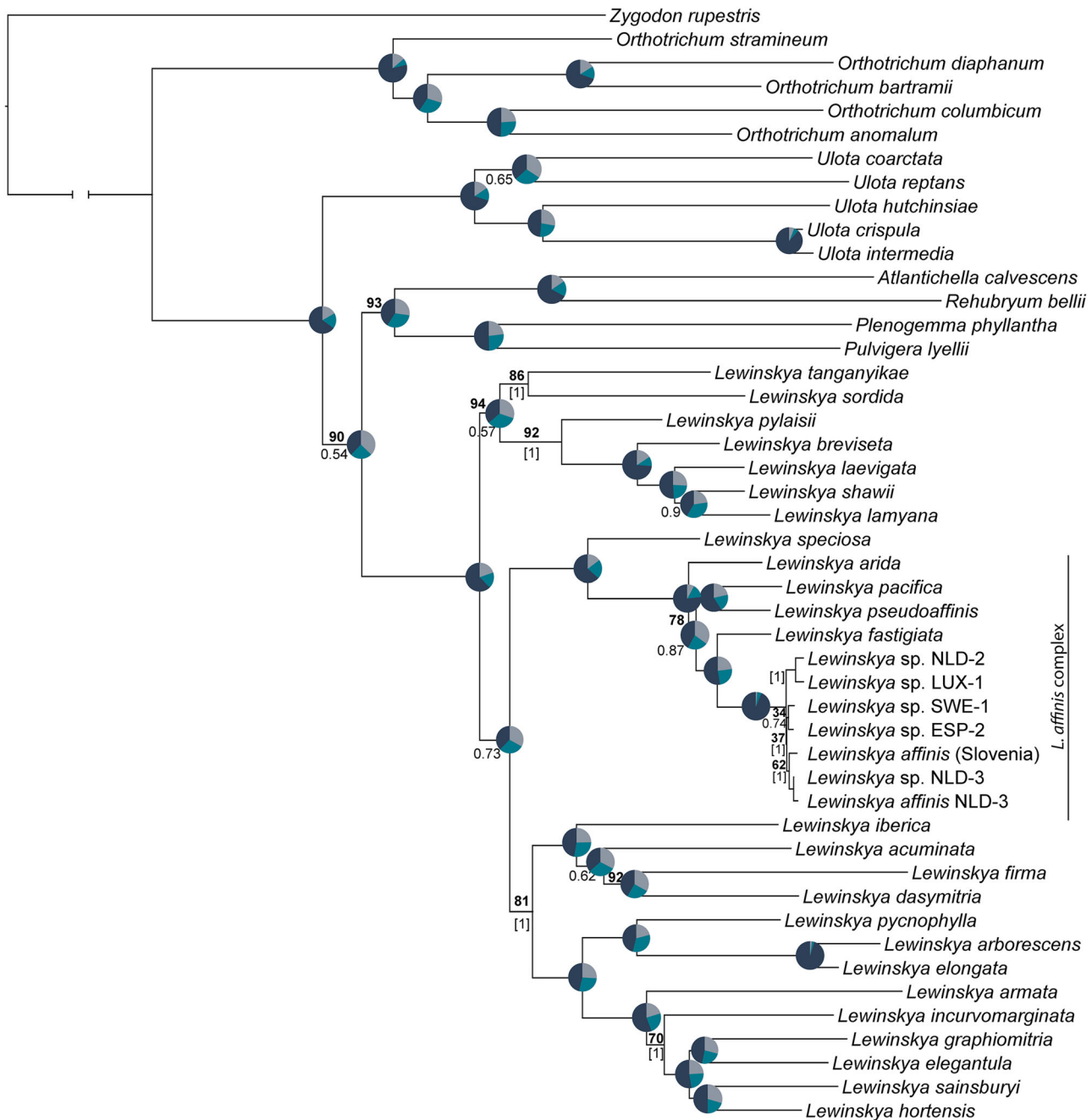
**Fig. 4.** **A**, Detail of a sporophyte from sample SWE-1. **B**, Upper part of the exothecium and peristome of a capsule from sample ESP-2.

## 4 Discussion

Occasionally, enigmatic individuals are found with puzzling characters that hamper a confident taxonomic ascription or classification, which can be a major problem for species delimitation. Traditionally, these specimens were assigned to a taxon based on morphological affinities. However, new molecular tools present potentially informative additional lines of evidence to assess their identity and evolutionary relationships. In bryology, such studies have been sporadically conducted (e.g., Skotnicki et al., 2001; Hedenäs et al., 2009; Sotiaux et al., 2009; Enroth et al., 2010; Bakalin et al., 2022) using a limited number of molecular markers. However, techniques, such as targeted enrichment, that allow the generation of vast amounts of genomic data sequenced on next-generation sequencing (NGS) platforms, can increase the information available for the interrogated samples and potentially strengthen the power and robustness of these analyses (e.g., Rokas et al., 2003; Vanderpoorten & Shaw, 2010; Young & Gillung, 2020, Šlipiko et al., 2022). These methods, combined with universal sets such as GoFlag 408 for flagellate plants or Angiosperms-353 (Johnson et al., 2019) for angiosperms, have been proven as a powerful tool in different fields (Dodsworth et al., 2019; Young & Gillung, 2020). This approach could represent a robust alternative to DNA barcoding (Blattner, 2016; Kadlec et al., 2017; Johnson et al., 2019; Larridon et al., 2020), opening up new opportunities for species identification (Erickson et al., 2008; Kane & Cronk, 2008; Sucher & Carles, 2008; Parks et al., 2009; Nock et al., 2011; Yang et al., 2013; Dodsworth, 2015; Li et al., 2015). Such methods may be particularly relevant and informative for bryophytes,

as they are characterized by their small size, sometimes have inconspicuous morphological features, and frequently lack sporophytes, challenging their identification. Moreover, target enrichment can be effective with small amounts of DNA—typical in bryology systematics, as it is advisable to use a single gametophyte shoot to avoid DNA contamination—and even with relatively old and degraded samples. This allows the use of ethanol-preserved tissues, stored DNA extractions, and even long-dried museum specimens (e.g., Faircloth et al., 2012; Straub et al., 2012; Bi et al., 2013; Guschanski et al., 2013; McCormack et al., 2013; Blaimer et al., 2016; Hart et al., 2016; Brewer et al., 2019; Forrest et al., 2019; Folk et al., 2021) and broadens the number of samples that can be analyzed, as exemplified by the 30-year-old sample included in this study (LUX-1).

Traditionally, *Lewinskya affinis* s.lat. was considered to exhibit great morphological variability, and its distribution was presumed to cover western North America, Southeast Asia, and East Africa, leading to discordant taxonomic treatments. However, Vigalondo et al. (2019) corroborated that the *L. affinis* complex comprises up to nine species, including *L. affinis* s.str., four species new to science, and two species previously synonymized. All our phylogenetic analyses recover all the anomalous moss samples together with *L. affinis* s.str. in a maximally supported clade, and relationships within this clade remain unresolved. This species is an important element of the epiphytic flora of western Europe (Vigalondo et al., 2019). Normal shoots of *L. affinis* s.str. were found to coexist with several of the anomalous moss samples. To analyze such cases, both a shoot from the anomalous moss and a shoot from *L. affinis* s.str. were selected from sample NLD-3 for sequencing. Our



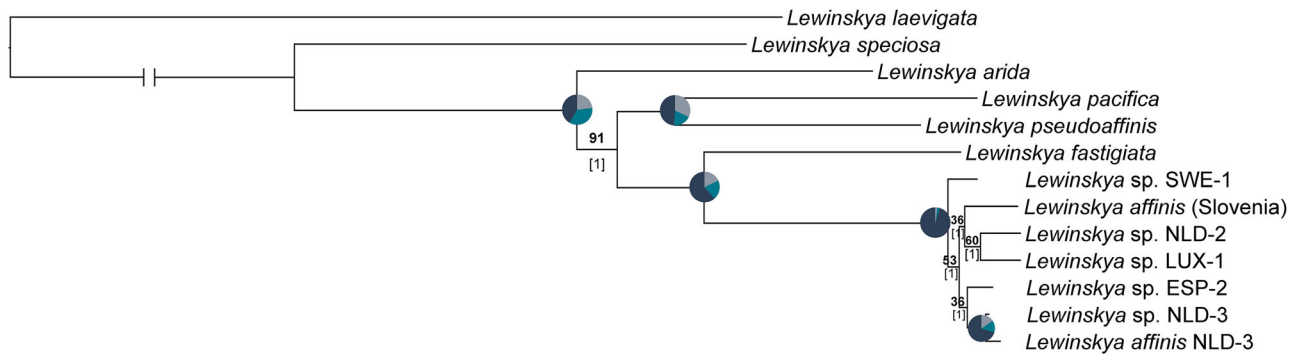
**Fig. 5.** Maximum likelihood (ML) phylogenetic reconstruction of Lewinskyinae using all 47 samples, inferred from a concatenated matrix of 385 loci. Values in bold above the branch indicate bootstrap support (BS) and values below the branch indicate local posterior probabilities (LPP) from the multiple species coalescent (MSC) analysis. If not indicated, the BS and LPP are over 95% and 0.95 respectively. Pie charts show quartet support, with dark blue representing loci agreeing with the main topology, light blue representing loci supporting the first alternative topology, and gray representing loci supporting a second alternative topology. When the ML and MSC topologies differ, the LPP value is indicated with square brackets, and the pie chart is not included.

results retrieved these two samples together with minimal sequence divergence, suggesting that all the problematic samples correspond to individuals of *L. affinis* with some kind of teratological or abnormal growth.

Despite intensive searching over two decades in numerous field campaigns targeting the subfamily Orthotrichoideae all

over the world, these anomalies of *L. affinis* have only been found a few times. Searching specifically for these forms in the field is extremely difficult since they occur sporadically and there is no obvious relationship between the abnormal forms and the environment where they were found. In addition, only a few anomalous specimens were found





**Fig. 6.** Maximum likelihood (ML) phylogenetic reconstruction of the *Lewinskya affinis* complex using 13 samples, inferred with the concatenated matrix of 372 loci, generated from the GoFlag 408 probe set, with flanking regions. Values in bold above the branch indicate bootstrap support (BS), and below the branch indicate local posterior probabilities (LPP). If not indicated, the BS and LPP values are maximum. Pie charts show quartet support, with dark blue representing loci agreeing with the main topology, light blue for loci supporting the first alternative topology, and gray for loci supporting a second alternative topology. When the ML and multiple species coalescent topologies differ, the LPP value is indicated with square brackets, and the pie chart is not included.

among herbarium collections. The studied collections of *L. affinis* and its related species, spanning its whole known distributional area (Vigalondo et al., 2019), suggest that these anomalous specimens are extremely rare.

Development drivers of these anomalous specimens remain unclear. Although environmental factors could potentially cause anomalous developments (Bell, 1991), no such relationship was found here, neither any sign of fungi nor other organisms' infections. It is also possible that the morphological abnormalities are the result of point mutations in growth-regulating regions of the genome. Such mutations could have occurred several times independently or, conversely, could have occurred once and spread. Although *L. affinis* lacks specialized asexual propagules and most of the anomalous samples do not develop sporophytes, they could spread over short to medium distances either by vegetative means (fragmentation) or by the occasional development of viable spores on unaffected capsules.

This is not the first case in which a moss with confusing morphology has been found to nest with little or no genetic differentiation within a previously described species. For example, *Platyhypnidium mutatum* Ochyra & Vanderp. and *Gradsteinia torrenticola* Ochyra, C. Schmidt & Bültmann were both described from a single locality in Germany (Ochyra & Vanderpoorten, 1999) and Tenerife (Ochyra et al., 1998), respectively. Later, Stech & Frahm (1999) and Werner et al. (2007) concluded that they were synonymous with *Platyhypnidium riparioides* (Hedw.) Dixon. *Hypnum heseleri* Ando & Higuchi (Ando & Higuchi, 1994) was also described as a new moss with an anomalous morphology. However, van Zanten & Hofman (1994) concluded that it was most probably a somatic mutant of *Hypnum cupressiforme* Hedw., which was later supported by other molecular studies (e.g., Kučera et al., 2019). This also seems to be the case for some of the *Thamnobryum* Nieuwl. species, which are known from one or very few localities (e.g., Hodgetts & Blockeel, 1992; Olsson et al., 2009).

Strong morphological dissimilarities and weak genetic differentiation are found in many other bryological studies (e.g., van Zanten et al., 1988; van Zanten & Hofman, 1994; Stech & Frahm, 1999; Stech et al., 1999; Shaw & Allen, 2000; Buryová & Shaw, 2005; Hassel et al., 2005; Werner et al., 2007; Olsson et al., 2009, 2012; Sotiaux et al., 2009; Cezón et al., 2010; Huttunen & Ignatov, 2010; Hedenäs et al., 2012; Maltseva et al., 2023). This may be due to different evolutionary rates in morphological traits and genetic markers (Brakefield, 2006; Hedenäs & Eldenäs, 2008; Roux et al., 2016), incomplete lineage sorting (Draper et al., 2015), introgression events or hybridization (Natcheva & Cronberg, 2004; Gustavsson et al., 2005; Hedderson & Nowell, 2006; Draper et al., 2007; McDaniel et al., 2010; Vilnet et al., 2012; Nieto-Lugilde et al., 2018a; Bakalin et al., 2022), or plasticity of the studied characters (Shaw & Allen, 2000; Vanderpoorten & Shaw, 2010; Nieto-Lugilde et al., 2018b). Recent divergence could also explain the lack of molecular differentiation. In such cases, sampling a higher proportion of the genome and making use of NGS technologies may offer more insight. Hedenäs & Eldenäs (2008), Sotiaux et al. (2009), and Vanderpoorten & Shaw (2010) suggested that a single or a few genes may be responsible for dramatic morphological changes. Indeed, some differences in morphological traits may not be the result of mutations in specific genes but could be caused by gene regulation in cis-regulatory elements that give rise to phenotypic novelties (Stern, 2000; Davidson, 2001; Brakefield, 2006; Wray, 2007; Carroll, 2008; but see Marand et al., 2023 for a review in plants).

Although genetic data are becoming increasingly abundant and new methods are available, many problems remain in inferring reliable phylogenetic trees, and ensuring that the data are free of contaminants or paralogs. Phylogenomic data sets are computationally demanding and methodologically complex, accurate tree reconstruction is not always straightforward, and there are many workflow possibilities to proceed. The accumulation

of sequence data in recent years has reduced stochastic errors in phylogenetic analysis but has increased systematic errors (Kapli et al., 2020; Lozano-Fernandez, 2022), such as lack of reproducibility (Magee et al., 2014). For this reason, efforts are needed to concur with methodologies. This has been partly observed in recent years (e.g., McKain et al., 2018; Bravo et al., 2019; Kapli et al., 2020; Young & Gillung, 2020; Lozano-Fernandez, 2022). However, there is no universal set of best practices, and the path to choose depends on the studied organisms and the questions posed (Lozano-Fernandez, 2022). In this context, this study could serve as an example to follow in similar case studies, where resolution is indispensable for the correct ascription of puzzling and problematic samples, especially when the study material is very scarce.

## 5 Conclusions

Our analyses of new NGS data demonstrate that the samples of the anomalous orthotrichaceous moss found throughout Western Europe correspond to a single species, *Lewinskya affinis* s.str., a common species in the epiphytic environments of the area. The morphological anomalies appear to be species-specific. However, the teratological development of the specimens, which sometimes affects only a few leaves and sometimes the gametophores as a whole, or even some sporophytes, does not appear to be based on a substantial genetic differentiation. This study demonstrates how NGS data, and specifically targeted enrichment combined with a universal probe, can be used for species identification in plants.

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## Conflict of Interest

The authors declare no conflict of interest.

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## Supplementary Material

The following supplementary material is available online for this article at <http://onlinelibrary.wiley.com/doi/10.1111/jse.13040/supinfo>:

**Table S1.** Details of samples used for final phylogenetic reconstructions.

**Table S2.** Detailed description of all known samples with individuals showing anomalous morphology. Samples are arranged in the order in which they were received or found, and thus studied.

**Table S3.** Loci summary statistics for the A) probe only full dataset supermatrix, and B) full sequences reduce dataset supermatrix, that were used for final phylogenetic reconstructions.

**Table S4.** Concordance factors for the A) probe only full dataset supermatrix, and B) full sequences reduce dataset supermatrix, that were used for final phylogenetic reconstructions. Dark blue indicates the node that groups both anomalous and *Lewinskya affinis* s.str. samples. Light blue indicates the nodes included within it.