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Molecular and morphological studies on the *Didymodon tophaceus* complex

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Abstract
Recently two Mediterranean species closely related to *Didymodon tophaceus* (Brid.) Lisa have been described in the genus *Didymodon* Hedw.: *Didymodon sicculus* M. J. Cano et al. and *Didymodon erosus* J. A. Jiménez & J. Guerra. The former has been proved to be widespread around the Mediterranean basin in recent years, and this study points to a considerable extension of its distribution area towards north, since it has also been discovered in the Netherlands. *D. erosus*, with its narrow morphological characterization, was only described four years ago and has hitherto only been known from the original localities in Spain. The molecular study based on nrITS sequence data of some selected specimens of all the three species suggests that *D. erosus* is much more frequent and widespread in Europe than supposed and, furthermore, is morphologically much more variable than previously described. A key for the identification of the three species, an amended description of *D. erosus* and a map showing the known distribution area are presented.

Keywords: Didymodon tophaceus, Didymodon sicculus, Didymodon erosus, *nrITS sequences, taxonomy, morphological studies, European distribution*

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Introduction

Didymodon sicculus M. J. Cano, Ros, García-Zamora & J. Guerra was described from south Spain by Cano et al. (1996). In the original publication, it was indicated that it is morphologically related to *Didymodon luridus* Hornsch. and *Didymodon tophaceus* (Brid.) Lisa. Jiménez (2006) confirmed this similarity and concluded that the prime important characters to distinguish *D. sicculus* from *D. luridus* despite their ecological similarities are the papillose upper laminal cells and the yellowish-green colour of the lamina with alcoholic KOH. This author distinguished *D. tophaceus* from *D. sicculus* by the elongated epidermal cells of the ventral side of the costa and its occurrence on vertical calcareous rock faces with seeping water or similarly humid places, whereas *D. sicculus* shows quadrate or shortly rectangular ventral epidermal cells on the costa and grows in dry habitats.

The distribution area of *D. sicculus* has been progressively widened around the Mediterranean basin. It has been summarized by Jiménez, Ros, et al. (2004) and later completed with new data of Cvetić and Sabovljević (2004) and Puglisi et al. (2004), extending its area through Algeria and Morocco in northern Africa; France, Greece, Italy (mainland and Linosa Island), Montenegro, Portugal (Azores) and Spain (mainland and Balearic and Canary Islands) in Europe; and Israel, Turkey and Yemen in western Asia.

Didymodon erosus J. A. Jiménez & J. Guerra has more recently been described from north-western Spain by Jiménez, Guerra, et al. (2004). No additional specimens were detected belonging to this species besides the detailed revision carried out by Jiménez (2006) of the genus *Didymodon* in Europe, North Africa and south-west and central Asia.

Morphologically, it was characterized by Jiménez, Guerra, et al. (2004)as follows: (1) rhizoidal tubers underground or on rhizoids in the lower part of the stem, (2) lanceolate to narrowly ovate-lanceolate leaves, (3) generally unistratose lamina, sometimes bistratose in patches, (4) margins that are markedly erose and papillose-crenulate in the upper middle part of the leaf, plane or lightly recurved from the

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base to 1/2 or 3/4 of the leaf, and (5) perichaetia that are terminal or on short lateral branches, generally in the bifurcation of two vegetative branches. They also considered *D. erosus* to be morphologically very closely related to *Didymodon sinuosus* (Mitt.) Delogne because of the similar stance of the leaves when moist and the erose and papillose-crenulate margins, and to *Didymodon tomaculosus* (Blockeel) M. F. V. Corley because of the rhizoidal tubers. Furthermore, it was observed that despite the morphological differences with *D. tophaceus*, both species share the same habitat.

D. tophaceus is a well-known species, very variable morphologically, for which many synonyms have been given and which continues to be found when some profound studies are carried out (Zander 1994; Jiménez et al. 2005). It is distributed worldwide (Jiménez 2006; Zander 2007).

The systematic studies based on nrITS sequences carried out in the genus *Didymodon* by Werner et al. (2005) withdraw support from most of the species relationships based on the morphology proposed for both *D. erosus* and *D. sicculus*. They observed that *D. sicculus* is not phylogenetically related to *D. luridus* and that *D. erosus* is neither closely related to *D. sinuosus* nor to *D. tomaculosus*. On the contrary, a highly supported monophyletic clade was obtained including *D. tophaceus*, *D. erosus* and *D. sicculus*.

Recently, some complex European specimens of the genus *Didymodon* have been found that could be related to this group of species. An example of this complexity can be observed in the note published by Frahm (2001), who made a call for help to identify a specimen found on an artificial slope. It was considered to be morphologically closely related to *D. tophaceus*, but showed some differences, such as the more acute apex, the shortly rectangular ventral cells on the costa, but not distinctly elongated (2:1), the partially bistratose lamina and the presence of two papillae on the laminal cells. Other problematic specimens have been detected mainly from central Europe.

The aims of this paper are (1) to verify the taxonomic value of the three species of the complex and (2) to establish the morphological differences between the taxa.

Materials and methods

Plant material

Seven populations of *D. erosus*, seven of *D. sicculus* and 12 of *D. tophaceus* were investigated for this study. Material was selected from different geographical areas, and specimens of uncertain identity were also included, such as the specimen mentioned in Frahm (2001). Identifications of problematic specimens were tentative and later confirmed or revised according to the molecular results. Most specimens were obtained from the herbaria of the authors and also from the Jan Kučera Herbarium in CBFS, which holds a large collection of the genus *Didymodon*. Based on the results of the earlier publication on the phylogenetic relationships within the genus *Didymodon* using nrITS data, *D. luridus*, *D. maximus* (Syed & Crundw.) M. O. Hill and *D. spadiceus* (Mitt.) Limpr. were chosen as outgroup species. Details of the geographical origin of the plant material, vouchers and GenBank accession number of the obtained sequences are given in Table I.

DNA isolation and amplification of ITS

DNA extraction. Total DNA was extracted by the NaOH extraction method described by Werner et al. (2002), in which 5 µl of crude NaOH extract was diluted by the addition of 45 µl of 100 mM Tris – 1 mM EDTA (pH 8.3) and stored frozen at –18°C until the PCR was carried out.

DNA sequencing. PCR was performed in an Eppendorf Mastercycler using 4 µl of the DNA solution in 50 µl final volume. The reaction mix contained the primers 18S (5′-GGAGAAGTCGTAACAAGGTTTCCG-3′), designed by Spagnuolo et al. (1999), and ITS4 (5′–TCCTCCGCTTATTGATATGC-3′; White et al. 1990), at a final concentration of 400 µM, in the presence of 200 µM of each dNTP, 2 mM $MgCl_2$, 2 units of Taq polymerase (Oncor Appligene), 1 µl BLOTTO (10% skimmed milk powder, 0.2% NaN_3 in water) and the buffer provided by the supplier of the enzyme. BLOTTO attenuates PCR inhibition by plant compounds (De Boer et al. 1995). Amplification started with 3 min denaturation at 94°C, followed by 35 cycles of 15 s at 94°C, 30 s at 50 C and 1 min at 72°C. A final extension step of 7 min at 72°C completed the PCR.

Amplification products were controlled on 1% agarose gels and successful reactions were cleaned with the help of the GenElute PCR Clean-Up Kit (Sigma-Aldrich). Cycle sequencing was performed with the Big Dyes Sequencing Kit (Perkin Elmer) using a standard protocol and the amplification primers. The annealing temperatures were set at 50°C. The reaction products were separated on an ABI Prism 3700 automatic sequencer (Perkin Elmer). When the sequence was not readable due to multiple different copies of the ITS region, the fragments were cloned using the GeneJet PCR Cloning Kit (Fermentas). Positive *Escherichia coli* colonies were directly transferred to a PCR tube, and the ITS region was amplified as described above, using the ITS primers.

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Table I. Collection identification, geographical origin and GenBank accession numbers for the taxa included in the molecular analysis.

Species	Geographical origin	Voucher specimen	GenBank accession ITS1/ITS2
Didymodon erosus Austria	Austria, Styria, Gulsen	*Köckinger* 94-1203b; Herb. H. Köckinger/MUB 28270; GZU	EU835148
Didymodon erosus France	France, Nice-Eze	*Skrzypczak 10080*; Herb. J. Kučera in CBFS	EU835143
Didymodon erosus Germany 1	Germany, Sachsen-Anhalt, Chörau	*Meinunger s.n, 19.10.1996,* Herb. L. Meinunger/ Herb. H. Köckinger/ MUB 28271	EU835145
Didymodon erosus Germany 2	Germany, Rheinland-Pfalz	*Frahm s.n.*; Herb. J. Kučera in CBFS	EU835146
Didymodon erosus Portugal	Portugal, Averio, Mata do Bussaco near Luso	*Väňa s.n.;* Herb. J. Kučera in CBFS	EU835144
Didymodon erosus Spain 1	Spain, Murcia, Sierra Espuña	*Romera Belchi & Pedreño Gonzalo s.n.;* MUB 6722	EU835136-EU835142
Didymodon erosus Spain 2	Spain, Salamanca, Linares de Riofrío	Werner et al. (2005)	AY437094
Didymodon sicculus Morocco	Morocco, Anti-Atlas, Tata	Werner et al. (2005)	AY437092
Didymodon sicculus Portugal	Portugal, Beira Litoral, Reserva Natural das Dunas de São Jacinto	*Cano s.n.* (Flora Briofítica Ibérica Project); MUB 13429	EU835132
Didymodon sicculus Spain 1	Spain, Cádiz, Grazalema	*Kučera 5628;* Herb. J. Kučera in CBFS	EU835123
Didymodon sicculus Spain 2	Spain, Almería, Salinas del Cabo de Gata	*Kučera 5333;* Herb. J. Kučera in CBFS	EU835126
Didymodon sicculus Spain 3	Spain, Murcia, Las Torres de Cotillas	Werner et al. (2005)	AY437091
Didymodon sicculus Netherlands 1	The Netherlands, Bravant	*van Melick 207857*; Herb. van Melick/MUB 28273	EU835124
Didymodon sicculus Netherlands 2	The Netherlands, Bravant	*van Melick 207878;* Herb. van Melick/MUB 28274	EU835125
Didymodon tophaceus Austria	Austria, Carinthia, Pörtschach	*Köckinger* 12353; Herb. H. Köckinger/MUB 28372/KL	EU835147
Didymodon tophaceus Czech Republic 1	Czech Republic, SE Moravia, Bílé Karpaty Mts.	*Hájek s.n.;* Herb. J. Kučera in CBFS	EU835130
Didymodon tophaceus Czech Republic 2	Czech Republic, C Bohemia, Český kras karst	*Kučera 2204;* Herb. J. Kučera in CBFS	EU835127
Didymodon tophaceus Czech Republic 3	Czech Republic, C Bohemia, Český kras karst	*Kučera 2203;* Herb. J. Kučera in CBFS	EU835128
Didymodon tophaceus Greece 1	Greece, central Greece and Euboea	Werner et al. (2004)	AY437093
Didymodon tophaceus Greece 2	Greece, Morfi-Omalí	*Cano s.n.*; MUB 17435	EU835134
Didymodon tophaceus Malta	Malta, Buskett	*Mifsud s.n.*; MUB 12542	EU835122
Didymodon tophaceus Spain 1	Spain, Granada, Sierra Nevada, Haza del Lino	*Zralíková s.n.*; Herb. J. Kučera in CBFS	EU835131
Didymodon tophaceus Spain 2	Spain, Almería, Los Molinos del Río de Aguas	*Kučera 5356*; Herb. J. Kučera in CBFS	EU835133
Didymodon tophaceus Spain 3	Spain, Murcia, río Alharabe	*Sánchez Fernández s.n.;* MUB 16209	EU835135
Didymodon tophaceus UK	UK, England, Cumbria	*Kučera 5861;* Herb. J. Kučera in CBFS	EU835129
Didymodon tophaceus USA	USA, Maine	*Allen 25750;* MO, MUB 18989	EU835149
Didymodon luridus 1	Morocco, Meknès-Tafilalt	Werner et al. (2005)	AY437097
Didymodon luridus 2	Greece, central Greece and Euboea, Delphi	Werner et al. (2004)	AY437098
Didymodon maximus	Ireland, Gleniff above Clogh	Werner et al. (2005)	AY437096
Didymodon spadiceus	Spain, Navarra	Werner et al. (2005)	AY437095

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Data analysis

The sequences were edited using Bioedit 5.0.9 (Hall 1999) and aligned manually. (The data are available from the corresponding author on request.) The aligned sequences were analysed using Maximum Parsimony (MP; Fitch 1971), Neighbor Joining (NJ) and Bayesian Inference. PAUP*4b10 (Swofford 2002) was used with MP and NJ as the optimality criterion, while MrBayes version 3.1 (Huelsenbeck & Ronquist 2001) served to calculate the posterior probabilities. Gaps were not treated as a fifth character state but were recodified as present or absent with the help of Seqstate (Müller 2005) using the modified complex coding option. In the case of MP, all characters were given equal weight and the heuristic search used the following settings: steepest descent off, TBR branch swapping, MULTREES on, 100 random-sequence additions saving an unlimited number of trees per replicate. All characters were equally weighted. A bootstrap analysis (Felsenstein 1985) with 1000 replicates was performed with the settings as mentioned. The NJ analysis was run with uncorrected pairwise distances as measure. Branching confidence was assessed using 1000 bootstrap replicates.

In the Bayesian analysis, 500,000 generations were run, sampling every 100th generation and using the following settings: Nst = 6, rates = gamma (general model of DNA substitution with gamma distributed rate variation across sites). These settings were chosen as the model that best fitted the data according to the Akaike Information criterion by Modeltest (Posada & Crandall 1998). Based on empirical evaluation, burn-in (the number of starting generations discarded from further analysis) was set at 100,000 generations. A 50% majority rule tree was constructed using the Sumt command of MrBayes. The tree was edited using TreeView version 1.6.6 (Page 1996).

Molecular results

The length of the ITS region for the ingroup species ranged from 763 to 777 bp. Within *D. sicculus*, the length variation was restricted to 763–766 bp, while *D. tophaceus* (771–777 bp) and *D. erosus* (772–776 bp) had slightly longer sequences. The length variation corresponds mainly to the ITS1 region (327–329 bp in *D. sicculus*, 334–335 bp in *D. tophaceus* and 335–339 bp in *D. erosus*). The ITS2 region was more constant with 277–278 bp in *D. sicculus* and *D. tophaceus* and 277–280 bp in *D. erosus*. The number of pairwise differences within species ranged from 0 to 8 for *D. sicculus*, 0–12 for *D. tophaceus* and 0–11 for *D. erosus*. These values exclude two specimens that are treated here (based on their morphology) as *D. tophaceus*, but which show sequences that are clearly different from the "normal" *D. tophaceus*. In the case of the only North American population available for this study, the number of pairwise differences was between 8 and 14 and in the other case (a central European population from Austria) between 12 and 21, compared with the other populations of *D. tophaceus*. For comparison we should mention that the pairwise differences between the outgroup species and the ingroup species ranged from 18 to 31.

One position of the alignment, an insertion of a "C", was exclusively shared by all samples of *D. erosus*. All samples of *D. sicculus* shared two "T→C" transitions not found in any other population, and all samples of *D. tophaceus*, with the exception of the two outliers mentioned above, had a "T" where all other populations show a "C" or a deletion.

The first impression based on the pairwise distances is confirmed by Bayesian Inference and NJ and MP analysis. Within the ingroup, there are three moderate to well supported clades separating the three species (Figure 1). The support for the *D. tophaceus* clade is the highest with values of 1.00 (Bayesian), 91% (MP) and 89% (NJ). The support for *D. erosus* (0.98/79/66) and *D. sicculus* (0.98/65/68) was lower. The exceptions are the two samples of *D. tophaceus*, which are separated by high-distance values from the other populations of this species and are not included in the *D. tophaceus* clade. The Bayesian, MP and NJ analyses therefore support the view that these specimens are isolated within the present dataset.

Morphological conclusions

D. sicculus and *D. erosus*, together with the well-known *D. tophaceus*, form a complex of "small species" that are clearly delimited molecularly but more difficult to separate at morphological level.

With the discovery of new specimens of *D. erosus*, it became clear that the species is more variable than previously described, and a redefinition of its morphological characters is necessary.

An amended description of *D. erosus* is presented below:

Didymodon erosus J. A. Jiménez & J. Guerra, Nova Hedwigia 78: 502. 2004

Plants 0.2–0.8(1) cm high, growing in dense turfs, olive-green. Rhizoidal tubers often present, underground or on rhizoids in the lower part of the stem, multicellular, uniseriate, straight or slightly curved, 35–65 × 12.5–25 μm, brown, smooth. Stems generally branched, hyalodermis absent, sclerodermis absent, seldom weakly differentiated, central strand absent or weakly differentiated; axillary hairs of two to six cells, with one to two brown

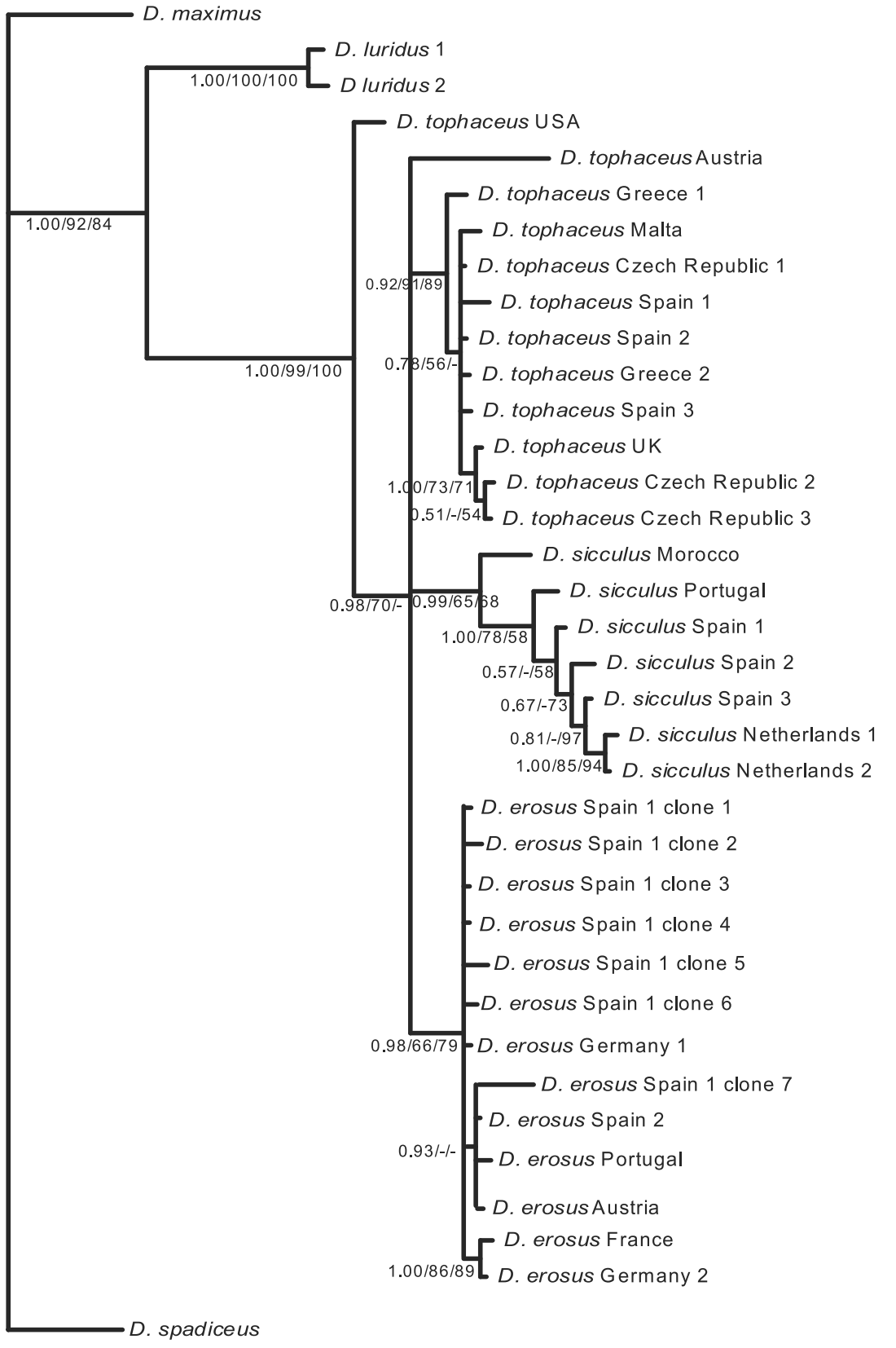

Figure 1. Bayesian phylogram illustrating the relationships between the three species of the *Didymodon tophaceus* complex. *Didymodon spadiceus*, *Didymodon maximus* and *Didymodon luridus* were used as outgroup species. Posterior probabilities as well as MP and NJ bootstrap values are indicated.

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basal cells and hyaline upper cells. Leaves erect-patent to incurved, occasionally twisted when dry, erect-patent to spreading when moist, deltoid to lanceolate, 0.5–1.5 × 0.2–0.4 mm; lamina unistratose, sometimes bistratose in patches, green or more rarely green-yellowish with KOH; apex acute; margins entire in the lower middle of the leaf, generally papillose in the upper middle, sometimes erose, plane or slightly recurved from base to $^{1}/_{2}$ or $^{3}/_{4}$ of the leaf, unistratose. Costa 35–75(90) μm wide at leaf base, excurrent in a thick mucro, percurrent or subpercurrent, ventral epidermal cells of the costa in the upper middle of the leaf quadrate or variously polygonal, papillose or smooth, dorsal epidermal cells of the costa in the upper middle of the leaf quadrate to rectangular, strongly papillose, occasionally smooth; costa in transverse section at leaf base semicircular, with two to five guide cells in one layer, zero to one layer of ventral stereids or one layer of substereids, one to two(three) layers of dorsal stereids, ventral epidermis differentiated, papillose or smooth, dorsal epidermis differentiated, strongly papillose, occasionally smooth. Upper and middle laminal cells rounded to variously polygonal, oblate or not, (5)8–17(22) × 5–12.5(15) μm, with one to two simple or bifurcate papillae per cell, sometimes low, thick-walled; basal cells quadrate to rectangular, 10–30(40) × 8–12.5 μm, smooth or sparsely papillose, lightly thin-walled. Gemmae absent on leaves. Dioicous. Perichaetia terminal or on short lateral branches, generally in the bifurcation of two vegetative branches, with three to four(five) archegonia; perichaetial leaves similar to vegetative leaves. Sporophyte unknown.

In order to facilitate the differentiation of the three species a key is presented below:

(1a) Leaves lingulate to oblong-lanceolate, often longer than 1.5 mm, with obtuse or rounded apex; lamina entirely unistratose; costa usually ending several cells below the apex, generally with two stereid bands in transverse section; ventral epidermal cells of the costa elongated in the upper middle of the leaf, more rarely shortly rectangular; stem with central strand and sclerodermis differentiated; rhizoidal tubers absent……*D. tophaceus*

(1b) Leaves lanceolate, ovate or deltoid, not longer than 1.5 mm, with acute or rounded apex; lamina unistratose or bistratose in patches; costa subpercurrent to shortly excurrent, generally without ventral stereids or more rarely with a small layer of substereids in transverse section; ventral epidermal cells of the costa in the upper middle of the leaf quadrate to shortly rectangular; stem without central strand and sclerodermis not or hardly differentiated, rhizoidal tubers often present……2

(2a) Leaves ovate, with rounded to broadly acute apex, margins recurved up to 3/4 of the leaf…. ……*D. sicculus*

(2b) Leaves deltoid to lanceolate, with rather narrowly acute apex, margins plane or more rarely slightly recurved from base to 1/2 or 3/4 of the leaf……*D. erosus*

The distribution of *D. erosus* is now much wider than known before (Figure 2), and includes four additional European countries: Austria, France, Germany and Portugal (see localities in Table I). In Spain the distribution has widened from north-west to south-east (see map in Figure 2). Also for *D. sicculus* the distribution area has widened northwards, being reported for the first time in the Netherlands.

As indicated in the key the most important morphological characters to differentiate these three species are: the leaf form (Figure 3A, 3B and 3C) which is lingulate to oblong-lanceolate in *D. tophaceus*; ovate in *D. sicculus* and deltoid to lanceolate in *D. erosus*; the leaf apex (Figure 3D, 3E and 3F) which is obtuse or rounded in *D. tophaceus*, rounded to broadly acute in *D. sicculus* and acute in *D. erosus*. The leaf margins are plane or more rarely slightly recurved from base to 1/2 or 3/4 of the leaf in *D. erosus*, while they are usually recurved up to $^{3}/_{4}$ of the leaf in *D. tophaceus* and *D. sicculus* (Figure 3A, 3B and 3C).

The erose margins, considered by Jiménez, Ros, et al. (2004) as an important diagnostic character to distinguish *D. erosus* from most of the other species of *Didymodon*, are not so marked in the new specimens studied in comparison to the original material (Figure 3C and 3F). Most of them only show small irregularities along the margins, as is also the case in *D. sicculus*, which can even be observed in the holotype (Cano et al. 1996, p. 403, figure 9) and the specimen MUB 5510 (Jiménez 2006, 274, figure 20g). Consequently, this character should not be considered as diagnostic for *D. erosus*.

Costal anatomy is also generally considered very important for the recognition of *Didymodon* species, particularly the presence or absence of ventral stereids (Jiménez 2006). Undoubtedly, this character can be used to separate *D. tophaceus* from *D. erosus* and *D. sicculus* (Figure 3G and 3H). In *D. tophaceus* there are usually two well-differentiated stereid bands, whereas in the other two species the ventral stereid band is frequently absent or, at most, a layer of substereids is present and the dorsal stereid band is often reduced, resulting in a homogeneous transverse section of the costa.

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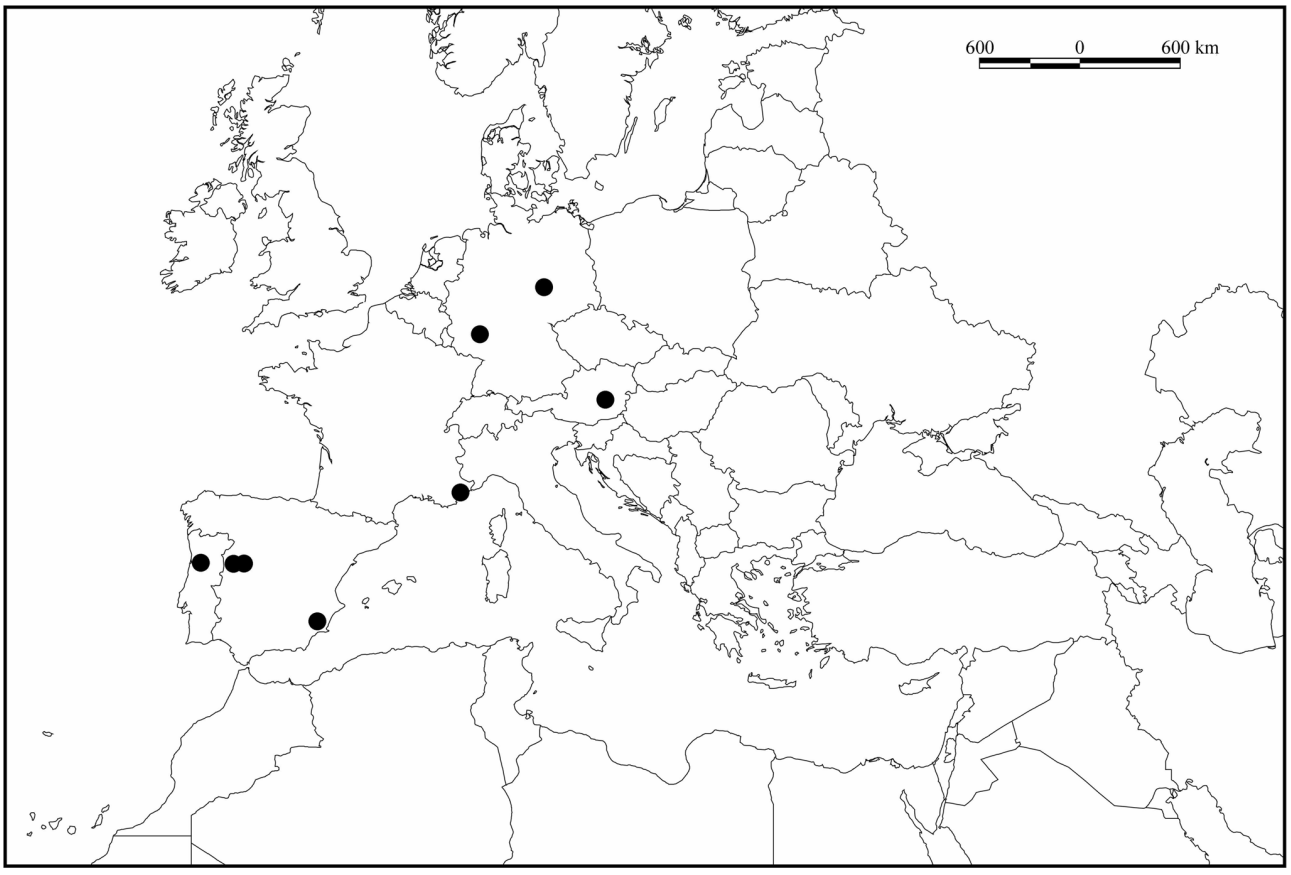

Figure 2. The known distribution of *Didymodon erosus*.

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Stem anatomy is frequently useful for distinguishing some *Didymodon* species (Jiménez 2006). This is the case for *D. tophaceus* which presents a differentiated central strand and a sclerodermis, while they are hardly or not differentiated in *D. sicculus* and *D. erosus*.

The shape of the ventral epidermal cells of the costa is considered to have high taxonomic value in the genus *Didymodon* (Saito 1975; Zander 1993; Jiménez 2006), and, in fact, this feature is constant in some species (elongated versus quadrate to rectangular). It was even considered by Zander (1993) as the most important feature to characterize the section *Fallaces*, although he accepted that two species, *Didymodon asperifolius* (Mitt.) H. A. Crum, Steere & L. E. Anderson and *D. tomaculosus*, belonging to this section, have quadrate or shortly elongate ventral cells. Nevertheless, Werner et al. (2005) showed that the section, even the group of species having elongated cells on the ventral side of the costa, is polyphyletic. In the three species studied here, this character seems to be relatively variable and alone cannot be used to segregate any species. In *D. tophaceus* it has been observed that the larger, upper leaves with rounded apices and rather thick costae show mainly elongated ventral costal cells, whereas the shorter, acutely pointed with narrower costae from lower parts of the same stems often possess isodiametrical ones down to midleaf. On the contrary, *D. erosus* and *D. sicculus* have quadrate to rectangular ventral costal cells. Hence, some overlapping is observed and, consequently, this character should only be used with caution in order to avoid confusion.

The presence of rhizoidal tubers has not been frequently described in the genus *Didymodon*. According to Jiménez (2006) and Zander (2007), they may be present in *Didymodon australasiae* (Hook. & Grev.) R. H. Zander, *D. erosus*, *Didymodon glaucus* Ryan, *Didymodon nevadensis* R. H. Zander, *D. tomaculosus* and *D. umbrosus* (Müll Hal.) R. H. Zander, but even in these species they are not always present or at least have not always been detected. The tubers are multicellular, normally uniseriate, brown, smooth, rounded to elongated, straight to variously curved, and are 30–145 μm in width. *D. tophaceus* has also been described as having rhizoidal tubers, at least in England (Side 1983), the Netherlands (Touw & Rubers 1989) and Ecuador (Arts & Sollman 1998). A revision of the corresponding material would be necessary to establish whether

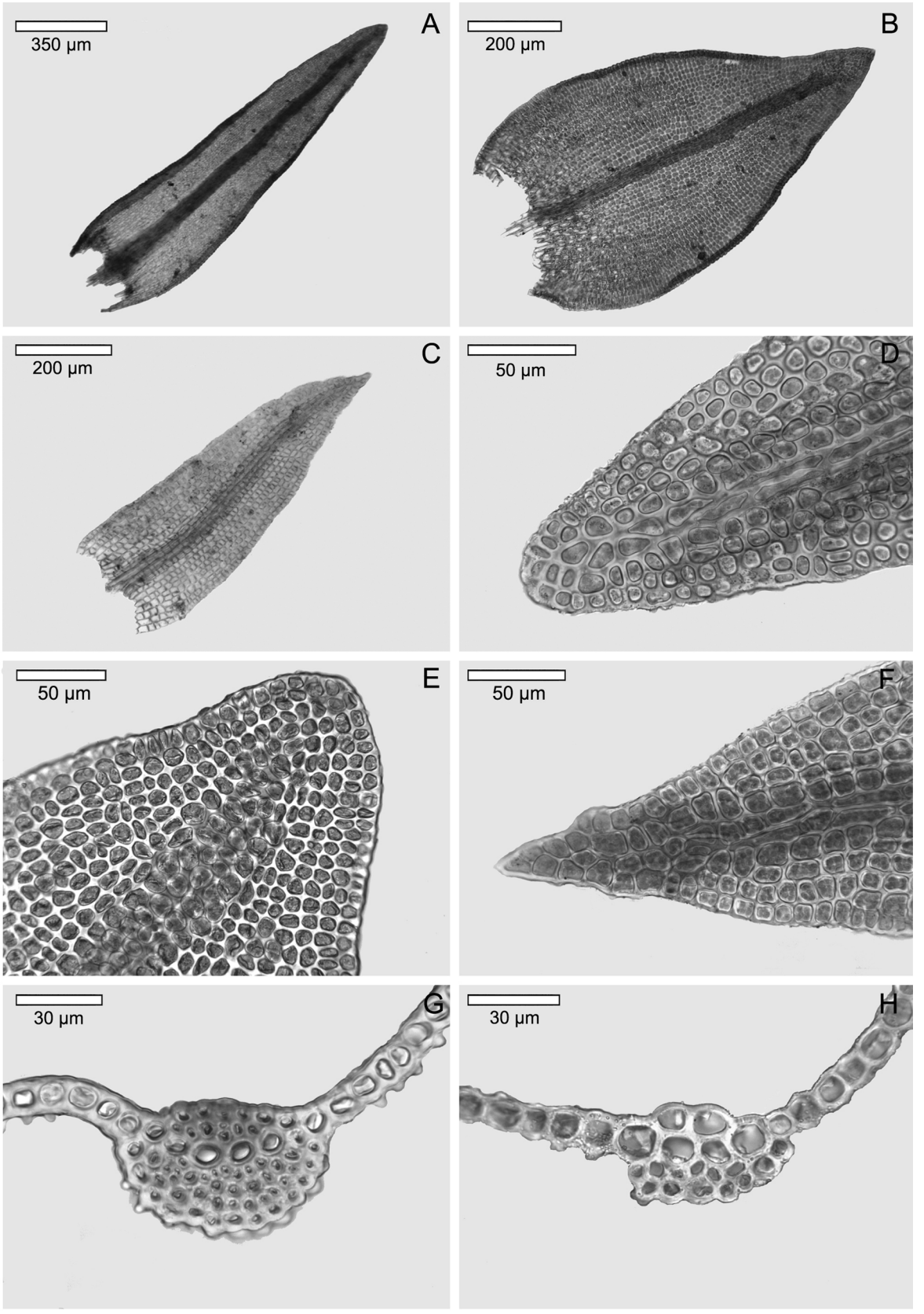

Figure 3. *Didymodon tophaceus* (from *Kučera 5861*): (A) leaf; (D) leaf apex and (G) transverse section of the costa at leaf base. *Didymodon sicculus* (from *van Melick 207878*): (B) leaf and (E) leaf apex. *Didymodon erosus* (from *Frahm s.n.*, in CBFS): (C) leaf; (F) leaf apex and (H) transverse section of the costa at leaf base.

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they could belong to *D. erosus* or *D. sicculus*. None of the *D. tophaceus* specimens studied in this work has rhizoidal tubers. Furthermore, not all the specimens that the molecular analysis has shown as belonging to *D. erosus* have been found to have rhizoidal tubers, although this is mostly due to the scarcity of the available material. We assume that the ability to develop rhizoidal tubers is genetically fixed in *D. erosus*. In this study, it was observed for the first time that *D. sicculus* can also have rhizoidal tubers (MUB 13425; van Melick 207857, 207878). The tuber morphology corresponds to that described for *D. erosus*.

Distinctive habitat preferences is a very important factor for differentiating these three species. *D. tophaceus* is clearly associated with wet calcareous substrates where water seeps or runs, whereas *D. sicculus* is strictly confined to dry soils. Unfortunately, the affinity to special habitat conditions is not very clearly defined in *D. erosus*, which has been observed to grow on wet rocks (tufa) but also in drier conditions such as rocky slopes, fissures of shaded walls, humid or dry soils and even on the gravel of forest paths. In general, this is an early pioneer which evolved in adaptation to the Mediterranean climate. The rhizoidal tubers allow the quick but ephemeral colonization of soil habitats and also survival of the dryness of the Mediterranean summers. In addition, they perfectly serve as spreading units to reach new localities. Both German collections originate from artificial habitats and may be interpreted as recent introductions. The single known Austrian population, growing on dry soil in steppe vegetation, however, should be seen as autochthonous, as it is associated there with a relict population of the Mediterranean fern *Notholaena marantae* (L.) R. Br.

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Discussion

The molecular data show with reasonable evidence that *D. erosus*, *D. sicculus* and *D. tophaceus* are distinct but closely related species. But, the data also show that there may be more cryptic or semicryptic species belonging to this complex. Our study almost exclusively includes European populations, but the data indicate that in the case of the widespread *D. tophaceus* there might be substantial differences between populations from different continents that would fall within the range of differences observed between the three species of this complex. Therefore, a worldwide approach might be very prolific. But even within the European samples, one distinctly outstanding population of *D. tophaceus* from Austria was found. Since these morphs seem to be rare and difficult to identify by morphology alone, a detailed study of this paradigm will need a sampling scheme which includes a very high number of specimens. But again, the data might be interesting to help understanding the speciation processes in this complex genus.

Alternatively, one might apply a very broad species concept, supported by the view that separating *D. sicculus* and *D. erosus* would leave the morphospecies *D. tophaceus* paraphyletic, although we do not believe that this argument is very profound. On one hand, *D. sicculus*, particularly, is clearly characterized by morphology and different ecology; on the other hand, it is widely assumed that in the early phases of speciation paraphyly is common (Avise 2000).

One interesting aspect concerning *D. sicculus* is the clear south-north gradient, in which most basal samples in the tree correspond to the southern specimens (especially Morocco) and the derived sequences to the northern ones (the Netherlands). Although the sampling scheme was not designed to answer biogeographical questions, this could mean that *D. sicculus* originated in North Africa and later spread northwards. This point of view is also supported to some degree by the ecology of this species, which clearly differs in its preference for dry habitats.

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