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A new species from the temporary ponds of southwest Portugal: *Helosciadium milfontinum*

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Abstract

The genus *Helosciadium* has six species of which three have been identified in Portugal: *H. nodiflorum*, frequent and abundant in much of the territory, and *H. inundatum* and *H. repens*, with a scattered distribution in Portugal. In the present study, a new species, *Helosciadium milfontinum*, a seriously threatened plant endemic, rare and scarce that grows in the temporary ponds of the Vicentinan Coastal District is described and illustrated. Morphological characters and molecular analysis of nuclear internal transcribed spacers (ITS) and plastid regions (*matK*, *rps16-trnK* and *trnL-F*) confirm the existence of this new taxon. Conservation status and taxonomic relationships of the new species are examined.

Keywords: Apiaceae, endemism, molecular markers, plant conservation, Portugal, taxonomy, temporary ponds

Introduction

The genus *Helosciadium* Koch (1824: 125) (Oenantheae tribe, Apiaceae family) is distributed throughout Europe, North Africa and Southwest Asia (Reduron 2007b). It is considered independent of *Apium* Linnaeus (1753: 264) (Apieae tribe) (see Reduron 2007a, b, Ronse *et al.* 2010, Downie *et al.* 2010) and includes six species: i) *H. bermejoi* (Llorens 1982: 3) Popper & M.F.Watson in Ronse *et al.* (2010: 187) - endemic to a small area of Menorca, Balearic Islands, Spain -; ii) *H. crassipes* Koch (1824: 125) - distributed in the Tyrrhenian environment: Corsica, Sardinia, Sicily, south of the Italian Peninsula and in the central area of North Africa -; iii) *H. inundatum* (Linnaeus 1753: 253) Koch (1824: 126) - which grows in western Europe and North Africa -; iv) *H. nodiflorum* (Linnaeus 1753: 251) Koch (1824: 126) -distributed across southern and western Europe, west from Asia and North Africa - , v) *H. muratianum* Maire (1937: 358) from the Republic of Chad (Equatorial Africa) but there is very little information about this taxon, making it is taxonomic evaluation difficult, and finally, vi) *H. repens* (Jacquin 1775: 34) Koch (1824: 126) - a plant with wide European distribution but scattered presence in the mountains of North Africa.

Helosciadium displays an unusual propensity for spontaneous hybridisation (e.g. Reduron 2007b, Stace *et al.* 2015). To date, four hybrid taxa have been formally described: (i) *Helosciadium* × *moorei* (Syme 1876: 20) Warren (1876: 20) – a hybrid between *H. nodiflorum* and *H. inundatum* (Desjardins 2016, O'Mahony 2016); (ii) *Helosciadium* × *clandestinum* Rita, Capó & Cursach (2016: 133) – a hybrid between *H. nodiflorum* and Menorcan endemic *H. bermejoi* (Rita *et al.* 2016, 2018); (iii) × *Beruladium procurrens* A.C. Leslie (2015: 790) – an intergeneric hybrid between *H. nodiflorum* and *Berula erecta* (Huds.) Coville (Desjardins *et al.* 2015), and recently (iv) *H.* × *longipedunculatum* (F.W.Schultz 1854: 237) Desjardins (2020: 37) – a hybrid involving the closely related species *H. nodiflorum* and *H. repens* (Desjardins *et al.* 2020).

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The presence of *Helosciadium repens* [synonym *Apium repens* (Jacq.) Lagasca (1821: 101)] in southwestern Portugal has been reported by Pereira Coutinho (1939) and Amaral Franco (1971). More recently, Flora-On: Flora de Portugal Interactiva (2014) indicates that this taxon should be included in Lista Vermelha da Flora Vascular de Portugal Continental (2019) under the threat category of Endangered (EN, "*Em Perigo"*). Furthermore, Knees (2003) reports the presence of this plant, under *Apium*, in southwestern Portugal with a detailed iconography of a specimen collected at "*Aguas da Moita, Odemira, Baixo Alentejo*" which morphology coincides with that presented in Flora-On: Flora de Portugal Interactiva (2014). Although previous studies have indicated that plants from southwestern Portugal should be identified as *Helosciadium repens*, preliminary data by Fernández Prieto *et al*. (2019), makes us doubt the correct identification of the taxon and leads us to hypothesize that it represents a new taxon.

The main goal of our study is to conduct a morphological and molecular study based on nuclear internal transcribed spacers (ITS) and plastid regions (*matK*, *rps16-trnK* and *trnL-F*) of all the described taxa of the genus *Helosciadium* and investigate the taxonomic status of this plant from southwest Portugal, and assess its possible hybrid origin using DNA barcoding.

TABLE 1. Samples of *Helosciadium* and related genera included in this study and their collections details. Herbaria codes: BC (Institut Botànic de Barcelona), FCO (University of Oviedo), SALA (Universidad de Salamanca).

Taxon	**Sample-Code**	Locality; Collector; Herbarium
Apium graveolens	APGR-a	"Porto das Barcas, Vila Nova de Milfontes, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 9 m, 37°44'11.39"N / 8°47'49.22"O; M. Ceballos & J.A. Fernández Prieto; FCO-39263."
	APGR-b	"Praia de Porto Covinho, Concelho de Sines, Distrito de Setubal (Baixo Alentejo, Portugal), 8 m, 37°51'37.50"N / 8°47'38.30"O; M. Ceballos & J.A. Fernández Prieto; FCO-39264."
Helosciadium bermejoi	HEBE-a/b	"Punta de sa Font, Menorca (Islas Baleares, España), 28 m, 39°59'51.58"N / 4°13'51.77"E; J. Rita Larrucea & M. Capó; FCO-35283, BC-637411."
Helosciadium crassipes	HECRA-a/b	"Giara de Gesturi, Gesturi, Sud Sardegna (Sardegna, Italia), 576 m, 39°44'18.15"N / 9° 0'6.76"E; F. Mascia, FCO-39268."
Helosciadium inundatum	HEIN-a	"La Morgal, Llanera (Asturias, España), 160 m, 43°25'57.78"N / 5°49'49.75"O; H. Nava; FCO-25637."
	HEIN-b	"Charca en Chozas de Arriba, Chozas de Abajo (León, España), 890 m, 42°31'14.58"N / 5°42'48.45"O; H. Nava; FCO-39267."
Helosciadium milfontinum	HEMI-a	"Portugal, Distrito de Beja, Concelho de Odemira, Freguesia de Vila Nova de Milfontes: Charcos temporários das dunas do Malhão, praderas de Agrostis stolonifera, 47 m, 37° 44.824'N / 8° 47.956'O, 26 September 2018; C. Pinto-Cruz, M. Ceballos de Horna & J.A. Fernández Prieto; FCO-39253."
	HEMI-b	"Charcos das dunas do Malhão, Vila Nova de Milfontes, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 47 m, 37°44'49.44"N / 8°47'57.37"O; C. Pinto-Cruz, M. Ceballos & J.A. Fernández Prieto; FCO-39260."
	HEMI-c	"Charcos das dunas do Malhão, Vila Nova de Milfontes, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 47 m, 37°44'47.85"N / 8°47'56.96"O; C. Pinto-Cruz, M. Ceballos & J.A. Fernández Prieto; FCO-39258."
	HEMI-d	"Charcos das dunas do Malhão, Vila Nova de Milfontes, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 49 m, 37°44'51.10"N / 8°47'45.11"O; C. Pinto-Cruz, M. Ceballos & J.A. Fernández Prieto; FCO-39259."
	HEMI-e	"Nascedios, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 31 m, 37°40'36.86"N / 8°47'20.94"O; C. Pinto-Cruz, M. Ceballos & J.A. Fernández Prieto; FCO-39256."

......continued on the next page

TABLE 1. (Continued)

Taxon	**Sample-Code**	Locality; Collector; Herbarium
Helosciadium nodiflorum	HENO-a	“Barrio de San Lázaro, Oviedo (Asturias, España), 239 m, 43°21’8.65”N / 5°50’35.33”O; M. Ceballos & J.A. Fernández Prieto; FCO: 39252.”
	HENO-b	“Nascedios, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 31 m, 37°40’37.38”N/ 8°47’21.43”O; C. Pinto-Cruz, M. Ceballos & J.A. Fernández Prieto; FCO-39261.”
	HENO-c	“Base de los acantilados de la playa de Almograve, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 10 m, 37°39’9.46”N /. 8°48’06.70”O; C. Pinto-Cruz, M. Ceballos & J.A. Fernández Prieto; FCO-39262.”
	HENO-d	“Porto das Barcas, Vila Nova de Milfontes, concelho de Odemira, Distrito de Beja (Baixo Alentejo, Portugal), 1 m, 37°44’11.39”N / 8°47’49.22”O; C. Pinto-Cruz, M. Ceballos & J.A. Fernández Prieto; FCO-39265.”
	HENO-e	“Allence, Pravia (Asturias, España), 71 m, 43°28’12.52”N / 6° 9’45.26”O; A. Bueno & J.A. Fernández Prieto; FCO-39266.”
Helosciadium repens	HERE-a	“Puertos de la Ballota, Lena (Asturias, España), 1670 m, 42°59’41.61”N / 5°52’30.13”O; A Bueno & J.A. Fernández Prieto; FCO: 39251.”
	HERE-b	“La Orbada (Salamanca, España), 817 m, 41° 6’15.39”N / 5°28’42.40”O; X. Giráldez & E. Rico; SALA-146313.”
	HERE-c	“Alcañices (Zamora, España), 772 m, 41°40’10.14”N / 6°20’41.83”O; F. Navarro & C.J. Valle; SALA-8399.”

Material and methods

Plant material and sampling

Samples were collected across the Iberian Peninsula, Menorca (Balearic Islands) and Sardinia to include all the described taxa of the genus *Helosciadium*. An additional sampling effort was made in southwest of Portugal around Vila Nova de Milfontes, the area where the possible new taxon had been collected before (Table 1, Fig. 1). In the same Portuguese territories, we collected *H. nodiflorum,* as well as *Apium graveolens* Linnaeus (1753: 264), type species of *Apium*, to show the independence of both genera. Voucher specimens were collected, dried by pressing in absorbent paper, stored at room temperature, and kept at University of Évora Herbarium (UEVH) and Herbarium of the University of Oviedo (FCO). Herbarium codes follow Thiers (2020).

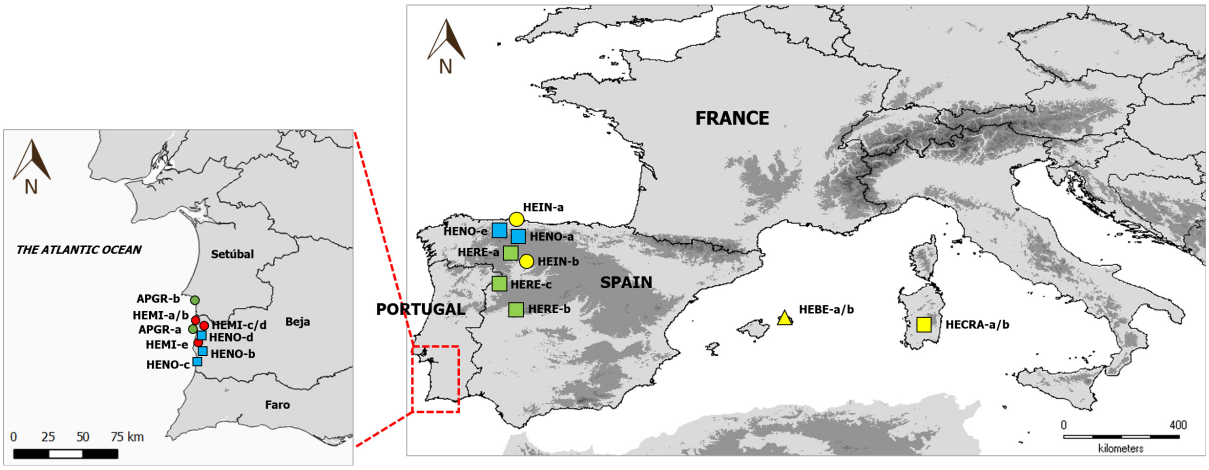

FIGURE 1. Distribution of the analyzed samples of *Apium graveolens* (APGR; green circle) and *Helosciadium*: *H. bermejoi* (HEBE; yellow triangle), *H. crassipes* (HECRA; yellow square), *H. inundatum* (HEIN; yellow circle), *H. milfontinum* (HEMI; red circle), *H. nodiflorum* (HEN; blue square) and *H. repens* (HERE; green square).

TABLE 2. List of species, sample code, and GenBank accessions for DNA sequences used in the molecular analysis [No data (-)].

Taxon; Code	GenBank accession			
	ITS	***matK***	***rps16-trnK***	***trnL-F***
Apium graveolens (APGR-a)	MT822069	MT822090	MT822111	MT822132
Apium graveolens (APGR-b)	MT822070	MT822091	MT822112	MT822133
Apium graveolens (APGR)	GQ379287	-	-	-
Hedera helix (HEHE)	AJ131227	AJ319074	DQ133864	FJ490767
Helosciadium bermejoi (HEBE-a)	MT822071	MT822092	MT822113	MT822134
Helosciadium bermejoi (HEBE-b)	MT822072	MT822093	MT822114	MT822135
Helosciadium bermejoi (HEBE)	AY353979	-	-	-
Helosciadium crassipes (HECRA-a)	MT822073	MT822094	MT822115	MT822136
Helosciadium crassipes (HECRA-b)	MT822074	MT822095	MT822116	MT822137
Helosciadium crassipes (HECRA)	AY360239	-	-	-
Helosciadium inundatum (HEIN-a)	MT822075	MT822096	MT822117	MT822138
Helosciadium inundatum (HEIN-b)	MT822076	MT822097	MT822118	MT822139
Helosciadium inundatum (HEIN)	AF164822	-	-	-
Helosciadium milfontinum (HEMI-a)	MT822077	MT822098	MT822119	MT822140
Helosciadium milfontinum (HEMI-b)	MT822078	MT822099	MT822120	MT822141
Helosciadium milfontinum (HEMI-c)	MT822079	MT822100	MT822121	MT822142
Helosciadium milfontinum (HEMI-d)	MT822080	MT822101	MT822122	MT822143
Helosciadium milfontinum (HEMI-e)	MT822081	MT822102	MT822123	MT822144
Helosciadium nodiflorum (HENO-a)	MT822082	MT822103	MT822124	MT822145
Helosciadium nodiflorum (HENO-b)	MT822083	MT822104	MT822125	MT822146
Helosciadium nodiflorum (HENO-c)	MT822084	MT822105	MT822126	MT822147
Helosciadium nodiflorum (HENO-d)	MT822085	MT822106	MT822127	MT822148
Helosciadium nodiflorum (HENO-e)	MT822086	MT822107	MT822128	MT822149
Helosciadium nodiflorum (HENO)	EF177709	-	-	-
Helosciadium nodiflorum (HENO)	KP871514	-	-	-
Helosciadium repens (HERE-a)	MT822087	MT822108	MT822129	MT822150
Helosciadium repens (HERE-b)	MT822088	MT822109	MT822130	MT822151
Helosciadium repens (HERE-c)	MT822089	MT822110	MT822131	MT822152
Helosciadium repens (HERE)	AY360241	-	-	-

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from 25 mg of dried tissue using DNeasy Plant Mini Kit (Qiagen, Germany). PCR reactions were performed as described in Cires *et al.* (2018) in 25 µL of final volume containing 25 ng of template DNA, 2.5 µL of 10X reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 U Taq DNA polymerase (Biotools, Madrid, Spain) and 0.08 µM of each primer [for nuclear ITS 17SE-26SE (Sun *et al.* 1994) and ITS1-ITS4 (White *et al.* 1990); for plastid sequences *matK* (de Vere *et al.* 2012); *rps16-trnK* (Lee & Downie 2006); and *trnL-F* (Shaw *et al.* 2005)]. DNA amplifications were performed as follows: initial denaturing at 94°C/4 min 30 cycles of denaturing at 94°C/1 min, 55°C/1 min and extension at 72°C for 1 min 30 s, and a final extension step at 72°C/10 min. PCR products were sequenced at the DNA Synthesis and Sequencing Facility Macrogen (Amsterdam, The Netherlands). The obtained sequences are deposited in GenBank (see Table 2 for accession numbers). Sequence data were assembled using ClustalW and edited with Geneious 7 (created by Biomatters; available from http://www.geneious.com/). Polymorphic sites in the sequences were IUPAC (International Union of Pure and Applied Chemistry) coded.

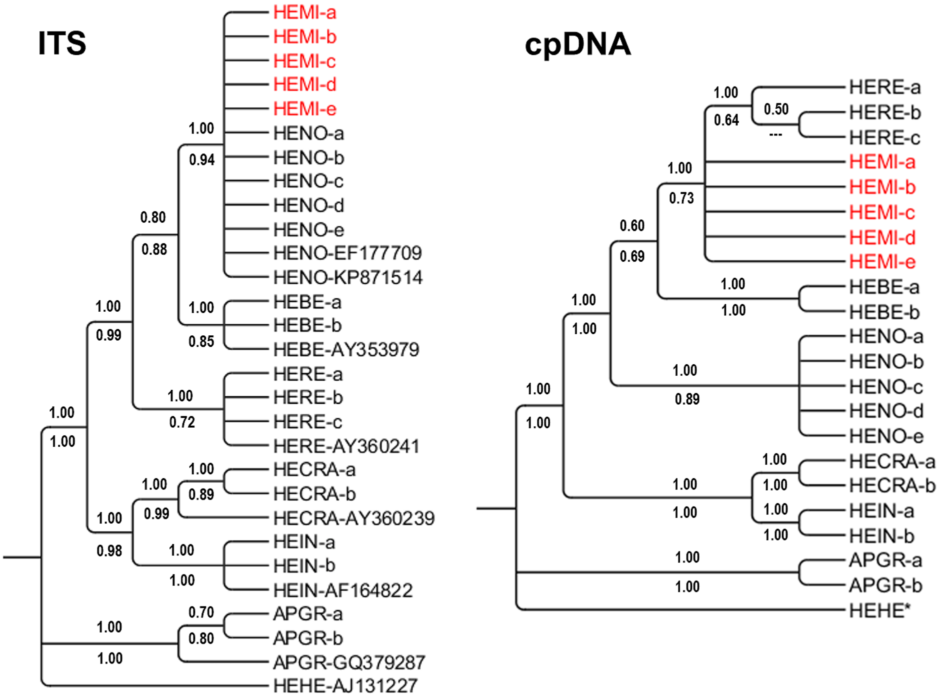

FIGURE 2. Phylogenetic consensus tree for *Helosciadium* species based on ITS sequences (left) and concatenated plastid regions (*matK*, *rps16-trnk* and *trnL-F*) (right) resulting from Maximum Parsimony and Maximum Likelihood analysis. Values along branches represent bootstrap (BS) values of ML (top) and MP (bottom), respectively. Dash symbol indicates the node is not present in the corresponding tree. APGR: *Apium graveolens*; HEBE: *Helosciadium bermejoi*; HECRA: *H. crassipes*; HEIN: *H. inundatum*; HEMI: *H. milfontinum*; HENO: *H. nodiflorum*; HERE: *H. repens*. *Hedera helix* (HEHE) was used as outgroup to root the tree (*concatenated sequences AJ319074–DQ133864–FJ490767).

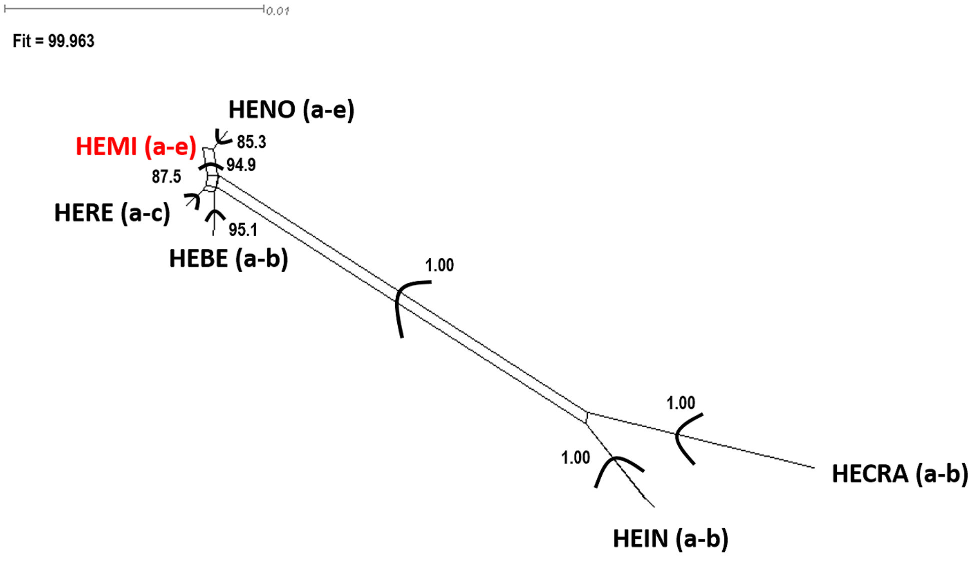

FIGURE 3. Phylogenetic network analysis (NeighborNet splits) based on concatenated plastid regions (*matK*, *rps16-trnk* and *trnL-F*). Numbers along branches are bootstrap values from 1000 replicates. HEBE: *Helosciadium bermejoi*; HECRA: *H. crassipes*; HEIN: *H. inundatum*; HEMI: *H. milfontinum*; HENO: *H. nodiflorum*; HERE: *H. repens.*

Phylogenetic and network analyses

Phylogenetic analyses of ITS sequences were performed using maximum parsimony (MP) and maximum likelihood (ML) methods, and conducted by a heuristic search using MEGA 8 (Kumar *et al.* 2018). The MP consensus tree was obtained using the Tree-Bisection-Regrafting (TBR). The evolutionary history was inferred by using the ML method and Tamura-Nei model (Tamura & Nei 1993). Both cases, the robustness of nodes was inferred from a bootstrap analysis (BS) of 1,000 replicates. Branches corresponding to the partitions reproduced in less than 50% bootstrap replicates were collapsed. Network analyses were constructed using the NeighborNet algorithm implemented in SplitsTree v.4.13.1 (Huson & Bryant 2006), applying uncorrected distances (also known as p-distances or Hamming distances). Bootstrap support for internal splits was calculated with 1,000 replicates. Fit values ranging from 0 to 100% indicate how well the information contained in the data was graphically represented. In all cases, *Hedera helix* Linnaeus (1753: 202) was used as outgroup.

Results

The characteristics of the nuclear (ITS) and plastid (*matK*, *rps16-trnK* and *trnL-F*) sequences used here for the genus *Helosciadium* are summarized in Table 3. The length of the aligned ITS sequences was 582 base pairs (bp), with 43 polymorphic sites (7.4%) and a mean GC content of 55.7%. Conversely, in the case of the combined plastid regions, the length of aligned sequences was 1,824 bp, with a mean GC content of 29.9%. Within the *Helosciadium* samples studied, the number of polymorphic sites was 179 (9.8%) while 1,645 sites were constant.

TABLE 3. Sequence characteristics of *Helosciadium* taxa obtained from the analysis of nuclear (ITS) and cpDNA (*matK*, *rps16-trnK* and *trnL-F*) sequences. Details for the ITS region include sequences sourced from GenBank.

	ITS	***matK***	***rps16-trnK***	***trnL-F***	**cpDNA concatenate**	**nuclear + cpDNA concatenate**
Length range (bp)	580–582	776	628–707	296–310	1713–1779	2293–2359
Aligned length (bp)	582	776	738	310	1824	2406
Constant characters	539	758	599	288	1645	2184
Polymorphic sites (%)	43 (7.4%)	18 (2.3%)	139 (18.3%)	22 (7.1%)	179 (9.8%)	222 (9.2%)
Number of indels (%)	44 (7.6%)	0 (0.0%)	875 (6.2%)	223 (3.8%)	1098 (3.2%)	1132 (2.5%)
Mean G+C content (%)	55.7	35.7	22.6	32.8	29.9	36.1

Phylogenetic trees resulting from the MP and ML analyses of ITS and cpDNA were very similar in topology and bootstrap support. Therefore, only one tree built on the derivative MP topology is represented (Fig. 2). The monophyly of the genus *Apium* is well supported (BS = 100%). Within *Helosciadium* two well-supported sister groups can also be recognized: the first one is formed by the species *H. inundatum* and *H. crassipes*, while the second group includes the rest of the species of the genus (*i.e. H. bermejoi, H. nodiflorum, H. repens*) and samples from southwest of Portugal currently identified as *H. repens* (indicated here with the acronym "HEMI"). However, it should be noted that position of these samples is different in the nuclear or chloroplast phylogeny. HEMI samples are the sister to *H. nodiflorum* in the ITS, meanwhile they have greater affinity with *H. repens* in the cpDNA tree. In addition to this, the monophyly of *H. bermejoi* is confirmed in all analyzes performed (BS = 100%).

The phylogenetic network analysis of concatenated sequences of ITS and cpDNA regions of *Helosciadium* (Fig. 3) shows the existence of two very well differentiated groups. The first one, consisting of samples of *H. inundatum* and *H. crassipes*, and a second group with the rest of the species (*H. bermejoi, H. nodiflorum, H. repens*) and the samples from southwest of Portugal (HEMI samples). Based on the genetic evidence presented above, we considered these samples represent a distinct entity within the genus *Helosciadium* and we describe it here as new species.

Taxonomy

Helosciadium milfontinum Fern.Prieto, Pinto-Cruz, Nava & Cires ***sp. nov.*** (Fig. 4)

Holotype: PORTUGAL. Distrito de Beja, Concelho de Odemira, Freguesia de Vila Nova de Milfontes: Charcos temporários das dunas do Malhão, praderas de *Agrostis stolonifera*, 47 m, 37° 44.824'N / 8° 47.956'O, 26 September

2018, *C. Pinto-Cruz, M. Ceballos de Horna & J.A. Fernández Prieto s/n.* Holotype: FCO-39253 (specimen in upper left corner, see Fig. 4). DNA sample code: HEMI-a.

HERBARIUM FCO
DEPARTAMENTO DE BIOLOGÍA DE ORGANISMOS Y SISTEMAS
UNIVERSIDAD DE OVIEDO

FCO 39253

Helosciadium milfontinum Fern.Prieto, Pinto-Cruz, Nava & Cires

Localidad: PRT: BAIXO ALENTEJO, Odemira, Vila Nova de Milfontes, charcos temporarios das dunas do Malhão

Cuadrícula U.T.M: 29SNB1768477771 **Altitud:** 47 m

Fecha: 26-IX-2018

Hábitat: Praderas de *Agrostis stolonifera*

Legit: C. Pinto-Cruz, M. Ceballos de Horna & J. A. Fernández Prieto

Deter: J. A. Fernández Prieto, C. Pinto-Cruz, H. Nava & E. Cires

Nota: Código de muestra: HEMI-a

FIGURE 4. Holotype of *Helosciadium milfontinum* (FCO-39253).

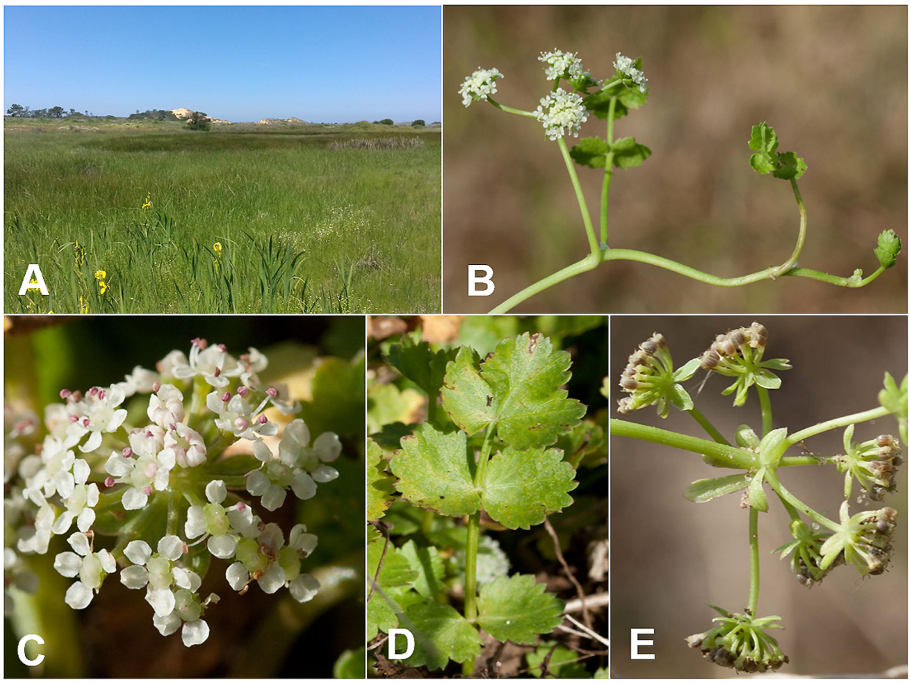

FIGURE 5. *Helosciadium milfontinum sp. nov.* (A) habit, (B) inflorescence, detail of pedunculated umbels; (C) inflorescence, (D) leaves, (E) umbels detail with 3 or more bracts. Photograph A by Carla Pinto Cruz; photographs B–E: modified images from Flora-On (*https://flora-on.pt/*) by Miguel Porto.

Iconography: Lam. 80, fig. a-j (Knees 2003) (sub. *Apium repens*). Photographs of *Apium repens* (Miguel Porto in Flora-On: Flora de Portugal Interactiva (2014) (see Fig. 5).

Diagnosis: *Helosciadium milfontinum* differs from *H. nodiflorum* by having the involucre of the umbel well developed, with 3 or more bracts; small fruit (c. 1 mm in length), ribs prominent and with well-marked vallecular vittae with subacute section. Differs from *H. repens* by having well-developed leaf sheaths and not rooting in all the nodes, becoming ascending in dense vegetation. The peduncles of the umbels are of similar length to the rays, longer than in *H. nodiflorum* and shorter than in *H. repens*. It has toothed or incised bracts and some toothed bracteoles (see Figs. 4 and 5).

Etymology: The epithet refers to the place where the plant grows, Vila Nova de Milfontes (Portugal).

Distribution: It grows exclusively in territories of Vicentinan Coastal District [Algarvese-Monchiquese Sector, Andalusian-Lusitanian Coastal Province, Mediterranean Region] (Rivas-Martínez *et al*. 2014).

Habitat: Found between 10–60 m. *Helosciadium milfontinum* grows mainly in grazing-resistant grasslands dominated by *Agrostis stolonifera* Linnaeus (1753: 62) (Pinto-Cruz *et al*. 2009) forming part of the community interest priority habitat "3170 *Mediterranean temporary ponds". Plants mostly grow among less dense formations of *A. stolonifera* and clearings. It also appears in communities dominated by *Eleocharis multicaulis* (Sm.) Desv. (1818: 74) (Pinto-Cruz *et al*. 2009), that form a mosaic with the previous ones and are integrated into the community interest habitat "3110 Oligotrophic waters containing very few minerals of sandy plains (*Littorelletalia uniflorae* Koch (1926: 26))" (see Council Directive 92/43/EEC, Council Directive 97/62/EC, European Commission 2013).

Phenology: Flowering from July to August, and fruiting from mid-August to early September.

Conservation status: Following the IUCN (2012, 2017) categories and recommendations, we suggest that *Helosciadium milfontinum* should be assessed as 'Critically Endangered' (CR), according to the following criteria: A1cd; B1ab(i,ii,iii,iv) + B2ab(i,ii,iii,iv). Further monitoring of the single known population is recommended to provide a more accurate conservation status. Although the new species occurs within two protected areas of Portugal (Parque Natural do Sudoeste Alentejano e Costa Vicentina and Costa Sudoeste Natura 2000 Site of Community Importance), *H. milfontinum* requires conservation action and a management plan to secure its long term survival. Moreover, it grows in two habitats of ecological interest: one priority habitat under the European Union Habitats' Directive "3170 *Mediterranean temporary ponds" and "3110 Oligotrophic waters containing very few minerals of sandy plains (*Littorelletalia uniflorae*)". Potential threats to the species and its habitat include the restricted distribution and agricultural intensification, respectively.

Other specimens examined:

Apium repens: PORTUGAL, Beja, Odemira, Vila Nova de Milfontes, *Life 219*, 37,7469941065 / -8,79931178844, 31 July 2018, *Erika Almeida & C. Pinto-Cruz*, UEVH-7427 FCO-39248. *Life 221*, 37,7477031405 / -8,79602569956, 31 July 2018, *Erika Almeida & C. Pinto-Cruz*, UEVH-7425 FCO-39249. *Life 220*, 37,7476260652 / -8,79772573794, 31 July 2018, *Erika Almeida & C. Pinto-Cruz*, UEVH-7426 FCO-39250.

Helosciadium milfontinum: PORTUGAL. Distrito de Beja, Concelho de Odemira, Freguesia de Vila Nova de Milfontes: Charcos temporários das dunas do Malhão, praderas de *Agrostis stolonifera*, 47 m, 37° 44.824'N / 8° 47.956'O, 26 September 2018, *C. Pinto-Cruz, M. Ceballos de Horna & J.A. Fernández Prieto*, FCO-39257.

Taxonomic remarks

Morphological and molecular data compiled in our study supports the recognition of a new species of *Helosciadium* (*H. milfontinum*) in southwestern Portugal. Based on ITS and cpDNA sequence data, we have showed that the new species has close affinity with *H. nodiflorum* and *H. repens*, respectively. The analysis of ITS sequence data is considered a useful resource for phylogenetic works in Apiaceae (Liu *et al.* 2014) and has been widely employed in studies of plant hybridization (e.g. Rita *et al.* 2018). On the other hand, cpDNA allows the detection of maternity and paternity roles because it is maternally inherited in almost all angiosperms. In this sense, the *rps16-trnK* intergenic spacer region of cpDNA, that we have used here, has been used in key studies of phylogeny in the Apiaceae family to establish maternity (e.g. Spalik *et al.* 2009, Desjardins *et al.* 2015).

Speciation via hybridization is the most likely origin for this new species. This mode of speciation is an important evolutionary mechanism in plants (Soltis & Soltis 2009) and it is likely to occur in plant species complexes in which species are evolutionarily young and sometimes not well differentiated, and the reproductive barriers between them are relatively weak (Guggisberg *et al.* 2009, Stace *et al.* 2015). Although our study has not investigated the timing for this event, sequencing data of *H. milfontinum* populations shows no evidence for current hybridization between *H. nodiflorum* and *H. repens*. Therefore, a past hybridization event between these or other closely related species, could explain the origin of this new species which is found in singular habitats different from those occupied by its putative parental species.

The uniqueness of *H. milfontinum* as an independent species is further supported when *H. milfontinum* samples were compared with those from all other known hybrids described in the genus *Helosciadium* (Fig. S1). Our study has clearly shown an ambiguous phylogenetic position of all putative hybrids described in *Helosciadium* (i.e. *H.* × *clandestinum* and *H.* × *longipedunculatum*) based on ITS and *rps16-trnK* sequences (see Table 4). For example, ITS sequences of putative hybrids of *H.* × *longipedunculatum* (Desjardins 2015, Desjardins *et al.* 2020) were visibly heterozygous (Fig. S2), meanwhile there was no ambiguous positions in the sequences of *H. milfontinum* from the different plant populations sampled from southwest of Portugal (i.e. HEMI samples).

Last, but not least, our phylogenetic results clearly discriminate between the genera *Apium* and *Helosciadium*, which supports the current view of various authors who segregate *Helosciadium* from *Apium* (e.g. Reduron 2007a, 2007b, Downie *et al*. 2010, Ronse *et al*. 2010). However, this position is not widely accepted (e.g. Tutin 1968, Knees 2003, Flora de Flora-On: Flora de Portugal Interactiva 2014). With regard to the morphological and genetic diversity exhibited across the genus *Helosciadium*, our study has confirmed that it is possible to differentiate the five species currently described in *Helosciadium* (i.e. *H. bermejoi*, *H. crassipes*, *H. inundatum*, *H. nodiflorum* and *H. repens*) based on morphological (Tutin 1968, Llorens 1982, Knees 2003, Reduron 2007b, Ronse *et al.* 2010) and molecular evidence (Hardway *et al.* 2004, Magee *et al.* 2008, Spalik *et al.* 2009, 2014, Rodríguez Canseco 2015, Desjardins *et al.* 2015, Desjardins 2015, 2016, Rita *et al.* 2016, 2018).

Key to species of *Helosciadium*:

The following dichotomous key is based on our study of *H. milfontinum* material and information gathered from Knees (2003), Reduron (2007b) and Ronse *et al*. (2010).

1. Dwarf acaule plant 1–4 cm tall, with stoloniferous stems; simple umbels .... ***Helosciadium bermejoi***

\- Caulescent plant of more than 4 cm tall, with submerged, prostrate, erect or ascending stems compound umbel 2

2. Leaves homomorphic 3

\- Leaves heteromorphic, those of the base divided into filiform linear segments, the upper ones in wide segments..........................5

3. Plants with sessile umbels or shortly pedunculate, peduncles shorter than rays, without involucre or if it appears formed 1 or 2 bracts; sheath leaves well developed and segments usually longer than wide... ***Helosciadium nodiflorum***

\- Plants with umbels more or less long pedunculated, involucre well developed and formed by 3 or more bracts; leaves with segments usually almost as wide as long 4

4. Plants rooting at all nodes; leaves without sheath; peduncles longer than rays; all bracts entire .... ***Helosciadium repens***

\- Procumbent or sometimes ascending plants and then not rooted in all nodes; sheath leaves slightly developed; umbels with peduncles equal or slightly longer than rays; some bracts incised o with toothed margins.................... ***Helosciadium milfontinum***

5. Fruiting pedicels thickened at the base, styles longer than the stylopodium, ovoid-subglobose fruit .... ***Helosciadium crassipes***

\- Fruiting pedicels not thickened at the base, styles shorter than the stylopodium, ovoid-elongate fruit... .... ***Helosciadium inundatum***

TABLE 4. Species-specific markers and mutation positions of ITS and *rps16-trnK* intergenic spacer regions for putative hybrids described in *Helosciadium* sequences with respect to *H. milfontinum* (samples HEMI-a – HEMI-e).

Taxon	**nrDNA ITS position**									**GenBank accession**
	12	**89**	**99**	**110**	**146**	**397**	**448**	**499**	**508**	
H. milfontinum (HEMI-a)	T	G	-	T	A	-	A	A	T	MT822077
H. milfontinum (HEMI-b)	T	G	-	T	A	-	A	A	T	MT822078
H. milfontinum (HEMI-c)	T	G	-	T	A	-	A	A	T	MT822079
H. milfontinum (HEMI-d)	T	G	-	T	A	-	A	A	T	MT822080
H. milfontinum (HEMI-e)	T	G	-	T	A	-	A	A	T	MT822081
H. x *clandestinum*-a	T	G	G	G	A	A	G	A	T	MT822153
H. x *clandestinum*-b	T	G	-	G	M	A	G	A	K	MT822154
H. x longipedunculatum-RXN1	A	A	-	G	A	-	G	G	T	MT108804
H. x longipedunculatum-RXN2 (copy1)	T	G	-	T	A	-	A	A	T	MT108805
H. x longipedunculatum-RXN2 (copy2)	A	A	-	G	A	-	G	G	T	MT108806
H. x longipedunculatum-RXN5 (copy1)	T	G	-	T	A	-	A	A	T	MT108807
H. x longipedunculatum-RXN5 (copy2)	A	A	-	G	A	-	G	G	T	MT108808

Taxon	**cpDNA rps16-trnK intergenic spacer position**							**GenBank accession**
	71-76	**85**	**86**	**177**	**186**	**418**	**555**	
H. milfontinum (HEMI-a)	TTATAT	A	T	T	G	T	G	MT822119
H. milfontinum (HEMI-b)	TTATAT	A	T	T	G	T	G	MT822120
H. milfontinum (HEMI-c)	TTATAT	A	T	T	G	T	G	MT822121
H. milfontinum (HEMI-d)	TTATAT	A	T	T	G	T	G	MT822122
H. milfontinum (HEMI-e)	TTATAT	A	T	T	G	T	G	MT822123
H. x *clandestinum*-a	TTATAT	A	T	T	G	C	G	MT822155
H. x *clandestinum*-b	TTATAT	A	T	T	G	C	G	MT822156
H. x *clandestinum*-H1	TTATAT	-	-	A	G	T	G	MF598292
H. x *clandestinum*-H2	TTATAT	-	-	A	G	T	G	MF598294
H. x *clandestinum*-H4	TTATAT	-	-	A	G	T	G	MF598296
H. x *clandestinum*-Y	TTATAT	A	T	T	G	C	G	MF598298
H. x longipedunculatum-RXN1	TTATAT	A	T	T	G	T	G	MT095026
H. x longipedunculatum-RXN2	TTATAT	A	T	A	G	T	G	MT095027
H. x longipedunculatum-RXN5	-	A	T	A	A	T	A	MT095028

Note: *H.* × *moorei* was not included due to the high number of changes presented for both markers.

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References

Amaral Franco, J. (1971) *Nova Flora de Portugal (Continente e Açores) Volume II*. Sociedade Astória, Lda., Lisboa, 660 pp.

Cires, E., Sanna, M., Vázquez, V.M. & Fernández Prieto, J.A. (2018) Genetics and conservation of rare and endemic plants: the case of *Genista sanabrensis* (Fabaceae) in the Iberian Peninsula. *Mediterranean Botany* 39: 77–87. https://doi.org/10.5209/MBOT.60078

Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora, O.J. L206, 22.07.92.

Council Directive 97/62/EC of 27 October 1997 adapting to technical and scientific progress Directive 92/43/EC on the conservation of natural habitats and of wild fauna and flora, O.J. L305, 8.11.1997.

de Vere, N., Rich, T.C., Ford, C.R., Trinder, S.A., Long, C., Moore, C.W., Satterthwaite, D., Davies, H., Allainguillaume, J., Ronca, S., Tatarinova, T., Garbett, H., Walker, K. & Wilkinson, M.J. (2012) DNA barcoding the native flowering plants and conifers of Wales. *PLoS One* 7 (6): e37945. https://doi.org/10.1371/journal.pone.0037945

Desjardins, S.D. (2015) *Evolutionary studies in subtribe Reynoutriineae (Polygonaceae). With contributions to the study of hybridisation in Helosciadium and Berula (Apiaceae) included as appendices*. Doctoral thesis, University of Leicester.

Desjardins, S.D. (2016) Molecular confirmation of *Helosciadium* × *moorei* (*H. inundatum* × *H. nodiflorum*; Apiaceae) from County Cork, Ireland. *New Journal of Botany* 6 (2–3): 90–97. https://doi.org/10.1080/20423489.2016.1271294

Desjardins, S.D., Leslie, A.C., Stace, C.A., Schwarzacher, T. & Bailey, J.P. (2015) Intergeneric hybridisation between *Berula erecta* and *Helosciadium nodiflorum* (Apiaceae). *Taxon* 64(4): 784–794. https://doi.org/10.12705/644.9

Desjardins, S.D., Shaw, A.C. & Judith, A.W. (2020) Hybridisation and introgression in British *Helosciadium* (Apiaceae). *British & Irish Botany* 2 (1): 27–42. https://doi.org/10.33928/bib.2020.02.027

Downie, S.R., Spalik, K., Katz-Downie, D.S. & Reduron, J.P. (2010) Major clades within Apiaceae subfamily Apioideae as inferred by phylogenetic analysis of nrDNA ITS sequences. *Plant Systematic and Evolution* 128 (1–2): 111–136. https://doi.org/10.1127/1869-6155/2010/0128-0005

European Commission, 2013. *Interpretation Manual of European Union Habitats*. Version Eur 28. DG Environment Nature ENV B.3, Brussels, 144 pp.

Fernández Prieto, J.A., Pinto Cruz, C., Nava, H., Bueno, A., Vázquez, V.M., Rita Larrucea, J., Capó Servera, M., Molina Abril, J.A. & Cires, E. (2019) Diversidad, filogeografía y conservación del género *Helosciadium* (Oenantheae, Apiaceae). In: Peñas de Giles, J. & Río Sánchez, J. del (Eds.) *Libro de resúmenes del 9º Congreso de Biología de la Conservación de Plantas, Granada*, pp. 96.

Flora-On: Flora de Portugal Interactiva (2014) *Sociedade Portuguesa de Botânica*. Available at www.flora-on.pt (accessed 28 July 2019).

Guggisberg, A., Mansion, G. & Conti, E. (2009) Disentangling reticulate evolution in an Arctic-Alpine polyploid complex. *Systems Biology* 58: 55–73. https://doi.org/10.1093/sysbio/syp010

Hardway, T.M., Spalik, K., Watson, M.F., Katz-Downie, D.S. & Downie, S.R. (2004) Circumscription of Apiaceae tribe Oenantheae. *South African Journal of Botany* 70 (3): 393–406. https://doi.org/10.1016/S0254-6299(15)30222-2

Huson, D.H. & Bryant, D. (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23 (2):

254–267.
https://doi.org/10.1093/molbev/msj030

IUCN (2012) *IUCN red list categories and criteria, ver. 3.1*. IUCN Species Survival Commission.

IUCN (2017) *Guidelines for Using the IUCN red list categories and criteria, ver.13*. IUCN Species Survival Commission.

Jacquin, N.J. (1775) *Florae Austriacae [...] Vol. III*. Typis Leopoldi Joannis Kaliwoda, Aulae imperialis typographi, Viennae, 55 pp.

Knees, S.G. (2003) *Apium* L. *In:* Castroviejo, S., Nieto Feliner, G., Jury, S.L. & Herrero, A.L. (Eds.) *Flora Iberica. X. Araliaceae-Umbelliferae*. Real Jardín Botánico, C.S.I.C., Madrid, pp. 269–275.

Koch, W.D.J (1824) Generum plantarum Umbelliferae nova dispositio. *Nova Acta Academiae Caesareae Leopoldino-Carolinae Germanicae Naturae Curiosorum. Verhandlungen der Kaiserlich Leopoldinisch-Carolinischen Deutschen Akademie der Naturforscher* 12: 57–156.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* 35: 1547–1549.
https://doi.org/10.1093/molbev/msy096

Lagasca, M.J. (1821) *Amenidades Naturales de las Españas*. Ibarra, Impresor de cámara de S. M., Madrid, 111 pp.

Lee, C.S. & Downie, S.R. (2006) Phylogenetic relationships within *Cicuta* (Apiaceae tribe Oenantheae) inferred from nuclear rDNA ITS and cpDNA sequence data. *Canadian Journal of Botany* 84: 453–468.
https://doi.org/10.1139/b06-016

Linnaeus, C. (1753) *Species plantarum, exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas*. Holmiae, Impensis Laurentii Salvii [L. Salvius, Stockholm], 1231 pp.
https://doi.org/10.5962/bhl.title.669

Lista Vermelha da Flora Vascular de Portugal Continental (2019). Sociedade Portuguesa de Botânica, Associação Portuguesa de Ciência da Vegetação e o Instituto da Conservação da Natureza e das Florestas, I.P. http://listavermelhaflora.pt/. (Accessed 28 August 2019)

Liu, J., Shi, L., Han, J., Li, G., Lu, H., Hou, J., Zhou, X., Meng, F. & Downie, S.R. (2014) Identification of species in the angiosperm family Apiaceae using DNA barcodes. *Molecular Ecology Resources* 14 (6): 1231–1238.
https://doi.org/10.1111/1755-0998.12262

Llorens, L. (1982) Un nuevo endemismo de la Isla de Menorca: *Apium bermejoi*. *Folia Botanica Miscellanea* 3: 27–33.

Magee, A.R., van Wyk, B.E., Tilney; P.M & Downie, S.R. (2008) *Ezosciadium* (Apiaceae): a taxonomic revision of yet another early diverging South African apioid genus. *Plant Systematic and Evolution* 276: 167–175.
https://doi.org/10.1007/s00606-008-0086-z

Maire, R. (1937) Contributions à l'étude de la Flore de l'Afrique du Nord, Fascicule 25. *Bulletin de la Société d'Histoire Naturelle de l'Afrique du Nord* 28: 332–388 + pl. 31.

O'Mahony, T. (2016) A taxonomic comparison of *Helosciadium inundatum* (L.) W.D.J. Koch, *H. nodiflorum* (L.) W.D.J. Koch and their interspecific hybrid, *H.* × *moorei* (Syme) Druce (Apiaceae). *Irish Botanical News* 26: 15–21.

Pereira Coutinho, A.X. (1939) *Flora De Portugal (Plantas Vasculares): Disposta Em Chaves Dicotomicas*. Verlag Von J. Cramer, Lehre, 938 pp.

Pinto-Cruz, C., Molina, J.A., Barbour, M., Silva, V. & Espírito-Santo, M.D. (2009) Plant communities as a tool in temporary ponds conservation in SW Portugal. *Hydrobiologia* 634: 11–24.
https://doi.org/10.1007/s10750-009-9885-7

Reduron, J.P. (2007a) Ombellifères de France, vol 1. *Bulletin de la Société Botanique du Centre-Ouest Bull, Nouvelle Série, Numéro spécial* 26: 1–564.

Reduron, J.P. (2007b) Ombellifères de France, vol 3. *Bulletin de la Société Botanique du Centre-Ouest Bull, Nouvelle Série, Numéro spécial* 28: 1143–1727.

Rita, J., Capó, M. & Curach, J. (2016) *Helosciadium ×clandestinum* un nuevo híbrido aparecido en Menorca (Islas Baleares). *Flora Montiberica* 63: 130–136.

Rita, J., Capó, M., Moragues, E., Bota, J. & Curach, J. (2018) Hybridization processes in an introduced subpopulation of an endangered plant: Management strategies to guarantee the conservation of *Helosciadium bermejoi* (Apiaceae). *Journal for Nature Conservation* 41: 26–34.
https://doi.org/10.1016/j.jnc.2017.10.006

Rivas-Martínez, S., Penas, A., Díaz-González, T.E., del Río, S., Cantó, P., Herrero, L., Pinto Gomes, C. & Costa, J.C. (2014) Biogeography of Spain and Portugal. Preliminary typological synopsis. *International Journal of Geobotanical Research* 4: 1–64.

Rodríguez Canseco, J. (2015) *Uso de secuencias ITS para el análisis de la diversidad, filogenia y sistemática del género* Apium *L. (Apiaceae)*. Trabajo de Fin de Grado, Facultad de Biología, Universidad de Oviedo, Oviedo, 19 pp.

Ronse, A.C, Popper, Z.A., Preston, J.C. & Watson, M.F. (2010) Taxonomic revision of European *Apium* L. s. l.: *Helosciadium* W.D.J.Koch

restored. *Plant Systematic and Evolution* 287: 1–17.
https://doi.org/10.1007/s00606-010-0284-3

Shaw, J., Lickey, E., Beck, J.T., Farmer, S.B., Liu, W., Miller, J., Siripun, K.C., Winder, C.T., Schilling, E.E., Small, R.L. (2005) The tortoise and the hare II: relative utility of 21 noncoding chloroplast sequences for phylogenetic analysis. *American Journal of Botany* 92: 142–166.
https://doi.org/10.3732/ajb.92.1.142

Soltis, P.S. & Soltis, D.E. (2009) The role of hybridization in plant speciation. *Annual Review of Plant Biology* 60: 561–588.
https://doi.org/10.1146/annurev.arplant.043008.092039

Spalik, K., Downie, S.R. & Watson, M.F (2009) Generic delimitations within the *Sium* Alliance (Apiaceae Tribe Oenantheae) inferred from cpDNA rps16-5'trnk$^{(UUU)}$ and nrDNA ITS sequences. *Taxon* 58 (3): 735–748.
https://doi.org/10.1002/tax.583004

Spalik, K., Banasiak, L., Feist, M.A.E. & Doenie, S.R. (2014) Recurrent short-distance dispersal explains wide distributions of hydrophytic umbellifers (Apiaceae tribe Oenantheae). *Journal of Biogeography* 41: 1559–1571.
https://doi.org/10.1111/jbi.12300

Stace, C.A., Preston, C.D. & Pearman, D.A. (2015) The Hybrid Flora of the British Isles. Botanical Society of Britain & Ireland, London, 501 pp.

Sun, Y., Skinner, D.Z., Liang, G.H. & Hulbert, S.H. (1994) Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. *Theoretical and Applied Genetics* 89: 26–32.
https://doi.org/10.1007/BF00226978

Tamura, K. & Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* 10: 512–526.

Thiers, B (2020) Index Herbariorum: A global directory of public herbaria and associated staff. New York Botanical Garden's Virtual Herbarium.

Tutin, T.G. (1968) *Apium* L. *In*: Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M. & Webb, D.A. (Eds.) *Flora Europaea 2: Rosaceae to Umbelliferae*. Cambridge, Cambridge University Press, pp. 351–352.

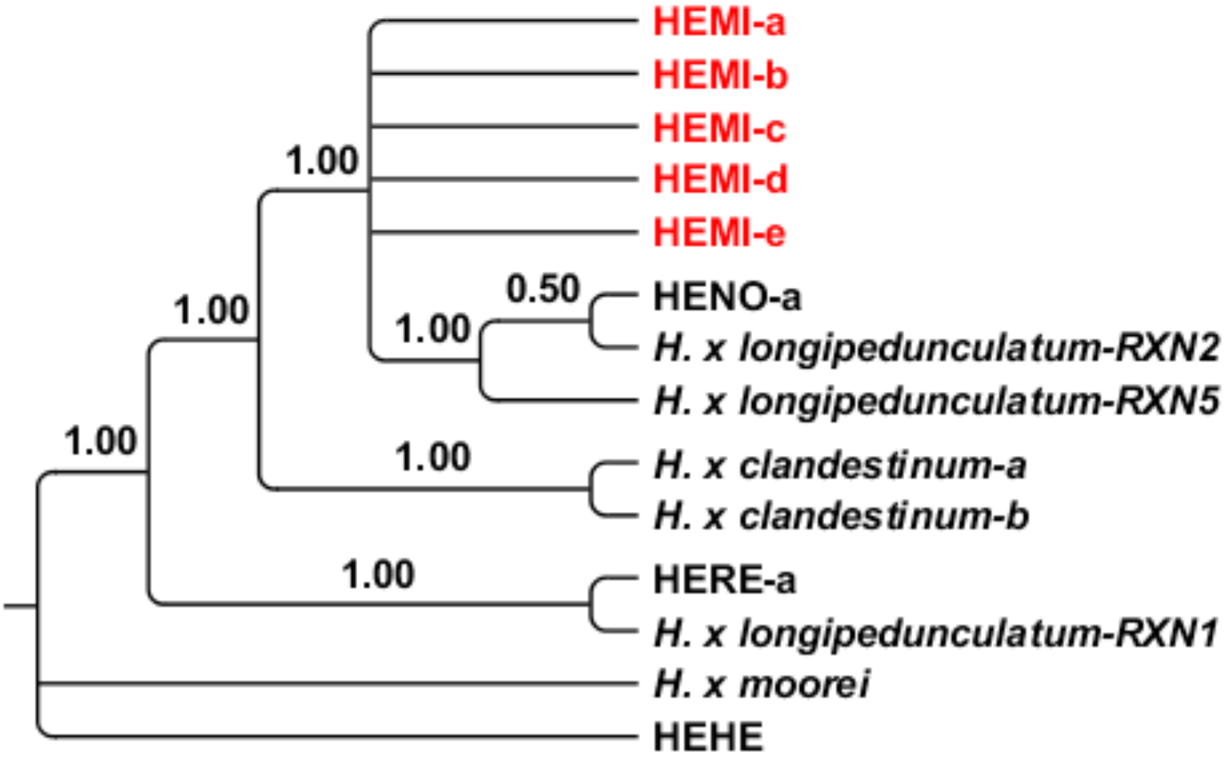

FIGURE S1. Phylogenetic consensus tree for putative hybrids described in *Helosciadium* based on concatenated analysis of ITS and cpDNA *rps16-trnK* intergenic spacer sequences using maximum parsimony analysis (MP). Values along branches represent bootstrap (BS) values of MP.

Sequences retrieved from Genbank are denoted with asterisk. GenBank accession numbers (ITS/*rps16-trnK*): HEMI-a (MT822077/MT822119), HEMI-b (MT822078/MT822120), HEMI-c (MT822079/MT822121), HEMI-d (MT822080/MT822122), HEMI-e (MT822081/MT822123), HENO-a (MT822082/MT822124), HERE-a (MT822087/MT822129), *H.* x *clandestinum*-a (MT822153/MT822155), *H.* x *clandestinum*-b (MT822154/MT822156), *H.* x *longipedunculatum*-RXN1 (MT108804*/MT095026*), *H.* x *longipedunculatum*-RXN2 (consensus MT108805*-MT108806*/MT095027*), *H.* x *longipedunculatum*-RXN5 (consensus MT108807*-MT108808*/MT095028), *H.* x *moorei* (KX513942*/KX513938*), HEHE (AJ131227/DQ133864).

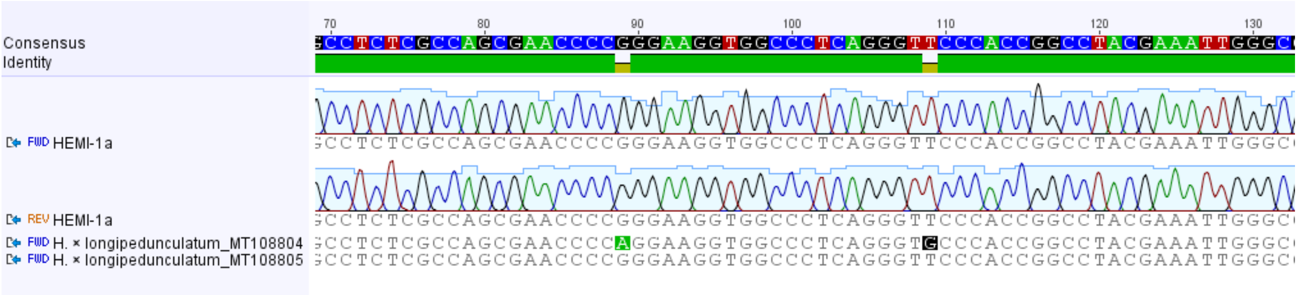

FIGURE S2. A partial view (70–130 bp) of ITS sequence chromatograms (forward and reverse) generated from *Helosciadium milfontinum sp. nov.* (present study) and *H.* × *longipedunculatum* described by Desjardins *et al.* (2020). *H.* × *longipedunculatum* was heterozygous for 5 SNPs identified between both taxa. GenBank accession numbers: MT822077, MT108804, MT108805.