Title: Intergeneric hybridisation between *Berula erecta* and *Helosciadium nodiflorum* (Apiaceae).

Authors: Stuart D. Desjardins¹, Alan C. Leslie², Clive A. Stace¹, Trude Schwarzacher¹ & John P. Bailey¹.

Addresses: 1) University of Leicester, Department of Biology, University Road, Leicester LE1 7RH, UK. 2) 109 York Street, Cambridge, CB1 2PZ, UK.

Author for correspondence: Stuart Desjardins (stuart.desjardins@gmail.com)

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ABSTRACT

A hybrid between Berula erecta and Helosciadium nodiflorum is reported from Chippenham Fen National Nature Reserve (NNR), Cambridgeshire, England. Parentage was investigated using chromosome counts, a maternally-inherited chloroplast marker (rps16-trnK), a biparentally-inherited nuclear marker (the ITS), and fluorescent in situ hybridisation (FISH) using labelled total genomic probes. Two parental genomes were identified in the putative hybrid (2n = 20), a maternal genome consisting of 9 chromosomes from B. erecta (2n = 18) and a paternal genome consisting of 11 chromosomes from H. nodiflorum (2n = 22). The implication of this hybrid for the taxonomy of the group is discussed, and a new hybrid genus is described: x Beruladium A.C. Leslie (= Berula Besser ex W.D.J. Koch x Helosciadium W.D.J. Koch). In overall appearance the hybrid resembles a small, creeping H. nodiflorum, and can be identified in the wild by a combination of characters: 1) the absence of a ring-mark on the petiole below the lowest pair of leaflets that is characteristic of pure Berula, 2) lower leaves with up to 5 pairs of leaflets, 3) peduncles varying from shorter than to longer than rays, 4) umbels with several usually untoothed bracts, 5) a relatively long stigma and style, and 6) the absence of ripe fruits. The detection of this taxon is of considerable interest and, as far as we know, it is the first confirmed intergeneric hybrid in the Apiaceae within Europe.
INTRODUCTION

Hybrids are unevenly distributed throughout the plant kingdom (Ellstrand & al., 1996). Some families have a disproportionately high number of hybrid taxa relative to their size, while others have only a few, or none at all (Whitney & al., 2010). The Apiaceae (umbellifers) are a large plant family where hybridisation is uncommon (Bell, 1971; Lovett-Doust & Lovett-Doust, 1982). Of the 50 or so native British species there are only three known hybrids from two genera, Helosciadium (Apium L. pro parte) and Heracleum L. (Stace, 2010). Intergeneric hybrids are particularly rare; we know of no confirmed cases in Europe.

Helosciadium (Apiaceae), formerly Apium section Helosciadium (W.D.J. Koch) Bab, is a small genus comprising five species: H. bermejoi (L. Llorens) Popper & M.F. Watson, H. crassipes W.D.J. Koch ex Rchb., H. inundatum (L.) W.D.J. Koch, H. nodiflorum (L.) W.D.J. Koch and H. repens (Jacq.) W.D.J. Koch (Ronse & al., 2010); three of which are native to the British Isles: H. inundatum, H. nodiflorum and H. repens (Stace, 2010). The genus is unusual within the Apiaceae as it displays a relatively high propensity for spontaneous hybridisation. In the British flora alone two hybrids are reported from the three native taxa: H. x moorei (Syme) Druce (= H. inundatum x H. nodiflorum), and H. repens x H. nodiflorum (Stace, 2010). However, identifying Helosciadium hybrids on the basis of morphology alone is often difficult as they can be mistaken for H. nodiflorum, which is widespread and diverse in form (Riddelsdell, 1914; 1917; Crackles, 1976; Walters, 1980). A random amplified polymorphic DNA (RAPD) analysis into a continuum of forms between H. nodiflorum and H. repens at Port Meadow Site of Special Scientific Interest (SSSI), Oxfordshire, England, also failed to confirm the presence of hybrids, but did not preclude them (Grassly & al., 1996).
Walters (1980) reported an unidentified *Apium* (= *Helosciadium*) specimen from Chippenham Fen NNR, Cambridgeshire, England, which was found growing near to a patch of typical *H. nodiflorum*; similar plants still exist at this site. The specimen was initially determined as putative *H. repens* but, due its misshapen pollen and sterile fruits, a potential hybrid origin was also suggested, *viz. H. repens x H. nodiflorum*.

An alternative proposal was that the Chippenham specimen was in fact a depauperate example of *Berula erecta* (Huds.) Coville, another British umbellifer, which is sometimes confused with *H. nodiflorum* in the vegetative state, or else an intergeneric hybrid between the two (Smith & Harding, 2001). *Berula erecta* is the only British representative of a widely distributed genus that occurs in Europe, Western Asia, Africa and North America. *Berula* was previously monotypic with *B. erecta sensu lato* as the only species, but seven taxa are now recognised: *B. bracteata* (Roxb.) Spalik & S.R. Downie, *B. burchellii* (Hook. f.) Spalik & S.R. Downie, *B. erecta sensu stricto*, *B. imbricata* (Schinz) Spalik & S.R. Downie, *B. incisa* (Torr.) G.N. Jones, *B. repanda* (Hiern) Spalik & S.R. Downie and *B. thunbergii* (DC.) H. Wolff (Spalik & al., 2009).

Previous cytological investigation has attempted to resolve the identity of the Chippenham specimen. Its somatic chromosome number was initially recorded as $2n = 19$, and this was taken to support its putative hybrid status (Walters, 1980), as *H. nodiflorum* is $2n = 22$ (IPCN, 1979-) and British *H. repens* was reported as $2n = 16$ at the time (Löve, 1972). However, the count for the Chippenham specimen was subsequently corrected to $2n = 20$ (Stace, 1984), and a consensus of $2n = 22$ reached for *H. repens* from mainland Europe (IPCN, 1979-). The Chippenham specimen therefore does not possess the 22 chromosomes expected of a *H. repens x H. nodiflorum* cross. *Berula erecta* is most
commonly reported as 2n = 18 (IPCN, 1979-), but there are no previous counts from the British Isles.

The current study aimed to identify putative hybrids collected from Chippenham Fen NNR, which are believed to represent material similar to that in Walters’ (1980) original description. A number of approaches were used to determine parentage, including: sequence data from a chloroplast marker, sequence data from a nuclear marker, chromosome counts and genomic in situ hybridisation (GISH). The chloroplast marker, \textit{rps16-trnK}, is maternally-inherited and was used to identify the seed-parent. The nuclear marker, the internal transcribed spacer (ITS), is biparentally-inherited and was used to identify all other lines of ancestry. Chromosome number was used to identify intermediate counts in the putative hybrids. GISH is a molecular cytogenetic technique in which labelled total genomic DNA is used as a probe for FISH to chromosomes (Schwarzacher & al., 1989), and was used to identify parental genomes in the putative hybrids.
MATERIALS AND METHODS

Plant material

Four collections (Table 8.1) were made from Chippenham Fen NNR, two species, *B. erecta* (7PR) and *H. nodiflorum* (10MSHED), and two putative hybrids (5MR & 10MR). The plants were cultivated under glass at the University of Leicester (UK) prior to molecular analysis and cytological investigation. Voucher specimens were made and deposited in LTR.

Molecular analysis

**DNA extraction, amplification and sequencing**

Total genomic DNA was isolated from silica-dried leaf material (20 mg) using the DNeasy Plant Mini Kit (Qiagen). Two markers, *rps16-trnK* and the ITS, were amplified by PCR. Amplifications were conducted in a reaction mixture containing: 1 x Buffer A (Kapa Biosystems), 1.5 mM MgCl₂ (Kapa Biosystems), 0.4 μM primers (forward and reverse; Sigma-Aldrich), 0.4 mM dNTP mix (Bioline), 0.5 U Kapa Taq DNA polymerase (Kapa Biosystems) and 100 ng template DNA, made up to 25 μL with ddH₂O (Sigma-Aldrich). For amplification of the ITS the master mix was additionally supplemented with 4% DMSO (Sigma-Aldrich), and for *rps16-trnK* with an extra 2.5 mM of MgCl₂ (total: 4 mM). The *rps16-trnK* intergenic spacer was amplified with the primers 5’ *exon* (*rps16*) (5’ TTT AAA ACG ATG TGG TAG AAA GCA 3’) and *trnK* (5’ TAC TCT ACC GTT GAG TTA GC 3’) (Lee & Downie, 2006), under the cycling conditions: 96 °C/02:00 + 30 x (96 °C/00:30, 50 °C/00:30, 72 °C/02:15) + 72 °C/07:00. The ITS region was amplified with the plant-specific primers, 17SE (5’ ACG AAT TCA TGG TCC GGT 3’) and 26SE (5’ TAG AAT TCC CCG GTT CGC TCG CCG TTA C’).
3’) (Sun & al., 1994), under the cycling conditions 97 °C/02:00 + 35 x (97 °C/01:00, 65 °C/00:45, 72 °C/01:15) + 72 °C/10:00.

PCR products were visualised by agarose gel electrophoresis. Amplicons were purified using the NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel), and sequenced directly. Nuclear ITS sequences from the putative hybrid specimens (5MR & 10MR) were also cloned. Cloning was conducted using the pGEM®-T Easy Vector System (Promega) and α-Select Competent Cells taken from *E. coli* (Bioline). Recombinant plasmids were selected for by blue-white screening, and the size of the insert determined by M13 colony PCR. *Plasmid DNA was isolated from cell cultures using the NucleoSpin® Plasmid kit* (Machery-Nagel). A minimum of two colonies were sequenced per accession. Sanger sequencing reactions were outsourced to Source BioScience (Nottingham, UK).

*Phylogenetic analysis* ----

Generated sequence reads were viewed, trimmed, blasted and aligned with Geneious R7 (created by Biomatters; available from [http://www.geneious.com/](http://www.geneious.com/)). Additional sequences were downloaded from the GenBank database (Supplementary information 8.7). Copies acquired from the putative hybrid specimens (5MR & 10MR) were investigated by blasting against the NCBI nucleotide database, directly comparing sequences and phylogenetic analysis. Maximum parsimony (MP) analysis was conducted using PAUP* 4.0 (Swofford, 2002). Topology searches were carried out using the branch and bound search strategy with the addition method FURTHEST. In order to estimate node support the inferred trees were resampled by bootstrapping (1000 replicates) using a heuristic search strategy (Felsenstein, 1985).

**Chromosome counts**
Root tips were collected from potted plants, pre-treated overnight @ 4 °C in a saturated aqueous solution of α-bromonaphthalene (Merck KGaA), fixed in 3:1 (v/v) ethanol: glacial acetic acid (24 hr @ 4 °C) and stored @ 4 °C until use. Next, root tips were hydrolysed in 5 N HCl (10 min @ RT) and transferred to 70% ethanol until use (Bailey & Stace, 1992). The root tips were then dissected, stained and squashed in aqueous 2% (w/v) aceto-orcein (Sigma-Aldrich; Darlington & Lacour, 1960). Cells were observed under bright-field on a Nikon Eclipse 80i microscope, somatic chromosome number recorded in at least 5 well-spread metaphases from different root tips, and a photograph of a representative cell taken using a Nikon DS-Qi1 digital camera and NIS Elements AR, version 3.2, software.

**Genomic in situ hybridisation (GISH)**

*Mitotic preparations* .---

Root tips were collected from the two putative hybrids (5MR & 10MR), as above. Pre-treatment chemical: 2 mM 8-hydroxyquinoline (Merck KGaA). Root tips were then enzyme digested in a citrate buffer containing: 1.8% (w/v) cellulase (72 U/ml; Calbiochem), 0.2% (w/v) ‘Onozuka RS’ cellulase (10 U/ml; Sigma-Aldrich) and 3% (v/v) pectinase (13.5 U/ml; Sigma-Aldrich) for 1 hr @ 37 °C, transferred to glass slides, dissected in 60% acetic acid and squashed (Schwarzacher & al., 1989). Preparations were preserved by freezing on dry ice, removing the coverslip and allowing to air-dry (Conger & Fairchild, 1953). Slides were then scanned for quality under phase contrast on a Zeiss Universal microscope, and stored @ -20 °C until use.

*Probe labelling* .---
Total genomic DNA (1 µg) of *B. erecta* (7PR) and *H. nodiflorum* (10MSHED) was labelled with digoxigenin-11-dUTP (Roche) or biotin-16-dUTP (Roche) using the Bioprime® Array CGH random priming kit (Invitrogen).

*Fluorescent in situ hybridisation.*

FISH followed the method outlined in Schwarzacher & Heslop-Harrison (2000). The probe mixture contained: 50% (v/v) deionized formamide (Sigma-Aldrich), 10% (w/v) dextran sulphate (Sigma-Aldrich), 0.125% (w/v) sodium dodecyl sulphate (SDS; Sigma-Aldrich), 1 µg/mL salmon sperm DNA (Sigma-Aldrich), 2 x saline-sodium citrate (SSC) and 200 ng of each probe, made up to 40 µl with ddH₂O (Sigma-Aldrich). No blocking DNA was added. Denaturation conditions were 10 min @ 90 °C for the probe mixture, and 7 min @ 72 °C for the chromosome preparations. Hybridisation conditions were 20 hr @ 37 °C. Experiments were carried out at a moderate stringency, the most stringent post-hybridisation wash being carried out in 20% formamide and 0.1 x SSC for 5 min @ 42 °C. Digoxigenin-labelled probe hybridisation sites were detected with fluorescein conjugated to a sheep anti-digoxigenin antibody (1 µg/ml; Roche), and biotin-labelled sites were detected with Alexa Fluor® 594 covalently attached to streptavidin (1 µg/ml; Invitrogen). Cells were counterstained with DAPI (4',6-diamidino-2-phenylindole; 4 µg/ml in McIlvaine’s buffer; Sigma-Aldrich). Preparations were observed using a Nikon Eclipse 80i fluorescent microscope under three Nikon filters UV-2E/C, B-2E/C and G-2E/C. Photographs were taken using a Nikon DS-Qi1 digital camera and NIS Elements AR, version 3.2, software. Images were later processed with Adobe Photoshop CS3, using only those functions that treat all pixels uniformly.
RESULTS

Chloroplast DNA marker

Amplified \textit{rps16-trnK} was around 1.8 kb in length. Average GC-content: 29.6%. A gene tree based upon \textit{rps16-trnK} sequence data and generated by MP analysis is shown in Fig. 8.1. The \textit{rps16-trnK} sequence of the two putative hybrids (5MR & 10MR) most closely matched \textit{B. erecta}. A nucleotide blast against the NCBI database showed a 99% similarity to \textit{B. erecta}. A direct sequence comparison with \textit{B. erecta} (7PR) gave a pairwise identity of 100%. The phylogenetic analysis also placed the copies in a \textit{Berula} clade with strong-support (99% bootstrap support, BS) and in a weakly-supported \textit{B. erecta} subclade (60% BS).

Nuclear DNA marker

Amplified ITS ranged from 846--866 bp in length. Average GC-content: 53.6%. The ITS of the putative hybrid accessions (5MR & 10MR) gave a mixed signal when sequenced directly, and subsequent cloning detected the presence of two main copies in each accession. A gene tree based upon ITS sequence data and generated by MP analysis is shown in Fig. 8.2. Copy 1 matched \textit{B. erecta}. Blasting showed 100% similarity to \textit{B. erecta}, a direct sequence comparison with \textit{B. erecta} (7PR) gave a pairwise identity of 100% and the phylogenetic analysis placed the copy in a \textit{B. erecta} clade with strong-support (100% BS). Copy 2 matched \textit{H. nodiflorum}. Blasting showed 100% similarity to \textit{H. nodiflorum}, a direct sequence comparison with \textit{H. nodiflorum} (10MSHED) gave a pairwise identity of 100% and the phylogenetic analysis placed the copy in a \textit{H. nodiflorum} clade with strong-support (97% BS). An additional copy (Copy 3) was detected in 10MR, which initially matched \textit{B. erecta} (0--334 bp), but then switched to \textit{H. nodiflorum} (335--847 bp). As the putative hybrid is presumed to be an F1 this chimeric
copy is likely to have recombined in vitro during PCR amplification (see Bradley & Hillis, 1997), and was uploaded to the GenBank database for future reference (KP871513), but not included in the phylogenetic analysis.

**Chromosome number**

Somatic chromosome number was acquired for all four accessions received as live material (Fig. 8.3). *Helosciadium nodiflorum* (10MSHED) was \(2n = 22\), *B. erecta* (7PR) was \(2n = 18\), and the two putative hybrids (5MR & 10MR) were \(2n = 20\).

**Genomic in situ hybridisation (GISH)**

Double-target in situ hybridisation of labelled total genomic probes to mitotic spreads identified two parental genomes in the putative hybrids, a *Berula* genome and a *Helosciadium* genome (Fig. 8.4). In cells at metaphase the *B. erecta* genomic probe hybridised to 9 chromosomes, while the *H. nodiflorum* genomic probe hybridised to the 11 other chromosomes, which were generally larger (Fig. 8.4B & E). Both probes hybridised along whole chromosome arms, often with distinct gaps at the centromeres, and showed little to no signal on the reciprocal chromosome set. Discrete genome domains with prominent chromocentres were visible in interphase nuclei, covering larger domains for *H. nodiflorum* compared to *B. erecta* (Fig. 8.4C & F). Repeating the experiments with reversed probe labels gave the same results (compare Fig. 8.4B & C with 8.4E & F).
DISCUSSION

The putative hybrid accessions from Chippenham Fen NNR were shown to be the result of intergeneric hybridisation between *B. erecta* and *H. nodiflorum*; as previously suggested by Smith & Harding (2001). Evidence for this hybrid combination came from the molecular and cytogenetic analyses. The maternally-inherited chloroplast marker identified a species of *Berula* as the seed-parent, the biparentally-inherited nuclear marker identified two parental species, *B. erecta* and *H. nodiflorum*, and GISH identified two parental genomes in the hybrids, a *Berula* genome and a *Helosciadium* genome.

Chromosome number

The chromosome numbers attributed to these plants have been the cause of much confusion. In particular *B. erecta*, which has been reported as: \( n = 6 \) (Bell & Constance, 1957), \( 2n = 18 \) (Scheerer, 1940), \( 2n = 18+0-2B \) (Lövkvist & Hultgård, 1999), \( 2n = 20 \) (B. Lövkvist in Weimarck, 1963) and \( 2n = 24 \) (Kochjarová, 1992). However, it is most commonly recorded as \( 2n = 18 \) (IPCN, 1979-), and we consider all other reports to be miscounts. *Berula erecta* from Chippenham Fen NNR was also \( 2n = 18 \), which is the first British record for this species. *Helosciadium nodiflorum* from Chippenham Fen NNR was \( 2n = 22 \), which is in line with all other British records for this species (*e.g.* Rutland, 1941; Hollingsworth & al., 1992; Dempsey & al., 1994). Unfortunately no live material of *H. repens* was available for cytological analysis in the current study. The hybrid specimens from Chippenham Fen NNR were confirmed as \( 2n = 20 \), which is an intermediate count and consistent with an intergeneric hybrid between *B. erecta* and *H. nodiflorum*.

Taxonomy

The evolutionary relationship of *B. erecta* and *H. nodiflorum* has been variously interpreted in past classifications of the Apiaceae. The first tribal classification of the
family by Sprengel (1820) placed the two taxa in the same genus, *Sium* L., within tribe Ammieae. Koch (1824), while keeping the taxa in the same tribe, split them over two genera, *Helosciadium* and *Sium* (including *Berula*), due to perceived differences in petal morphology. An overview of tribes and genera in the Apiaceae by Tutin (1962), which followed Drude (1898), has *Apium* (including *Helosciadium*) and *Berula* in subtribe Carinae of tribe Ammieae, but far apart, albeit only because of the degree of petal notching. A more recent review on the genera of the Apiaceae by Pimenov & Leonov (1993) placed the two genera in a large, heterogeneous tribe, Apieae, but did not propose any intratribal relationships.

The latest molecular phylogenetic schemes, using plastid and nuclear sequence data, have reported that *Berula*, *Sium*, *Helosciadium* and a small Holarctic genus, *Cryptotaenia* DC., form a strongly-supported, monophyletic clade within tribe Oenantheae, informally referred to as the *Sium* alliance (Downie & al., 2000, 2008; Hardway & al., 2004; Spalik & al., 2009). *Apium sensu stricto* (including the type: *A. graveolens* L.) does not fall within this clade and is well separated from it, belonging in tribe Apieae (Downie & al., 2000; 2010). Downie & al. (2008) further suggested that *Berula* and *Helosciadium* could be sister genera within the *Sium* alliance, although this hypothesised relationship was only weakly supported. The current consensus is that the four genera form a tetrachotomy; the relationships between them remaining largely unresolved (Spalik & al., 2009).

There are no obvious morphological synapomorphies for the *Sium* alliance and for this reason the subclades within, not the entire alliance, are given generic status (Spalik & al., 2009). *Berula*, *Sium* and *Helosciadium* do share a paludal/aquatic ecology and have similar vegetative morphologies with once-pinnate leaves, but *Cryptotaenia*
occurs in mesic habitats and has ternate leaves (Van Moorsel & Baudewijn, 2000; Spalik & al., 2009; Stace, 2010). More molecular studies, using a greater number of markers and/or whole genomes (nuclear and organelle), are needed to resolve the relationships within the *Sium* alliance, which remain unclear, although the detection of hybrids between *Berula* and *Helosciadium* does indicate a close relationship between the two genera.

Hybridisation and reticulate evolution

Hybridisation is traditionally regarded as a rarity in the Apiaceae (Bell, 1971; Lovett-Doust & Lovett-Doust, 1982). However, several recent phylogenetic studies have revealed incongruent plastid and nuclear datasets in a number of umbelliferous genera, e.g. *Osmorhiza* Raf. (Yoo & al., 2002), *Cicuta* L. (Lee & Downie, 2006), *Eryngium* L. (Calviño & al., 2008), *Oenanthe* L. (Spalik & al., 2009) and *Heracleum* L. (Yu & al., 2011), which has been interpreted, by some, as evidence for reticulate evolution in the family (Yoo & al., 2002; Calviño & al., 2008). It is therefore possible that hybridisation and introgression have played a more significant role in the evolutionary history of the Apiaceae than has been hitherto suspected, at least in some lineages. The demonstration here that vigorous (albeit sterile) hybrids can form between different genera, with an estimated date of divergence of *circa* 14 MYA (Spalik & Downie, 2006), can be taken to support this view. This is in addition to other interspecific hybrids known to occur within the family, such as those in *Heracleum* (Stewart & Grace, 1984; Jahodová & al., 2007). However, it must be stated that the evidence for modern hybridisation remains relatively scarce in the Apiaceae, when compared with many other plant families. Furthermore, the discordance between nuclear and chloroplast trees could have been caused by processes other than reticulation, such as incomplete lineage sorting and paralogous sampling, and
more in-depth studies using a greater number of unlinked nuclear loci are needed (Maddison, 1997; Yu & al., 2011).

Case for a new hybrid genus

The *Berula x Helosciadium* hybrid is a creeping, perennial plant with stoloniferous stems, which root at most nodes; forming large patches and with the potential to become locally abundant. The hybrid appears to be sterile, but can propagate vegetatively by means of lateral shoots, which are readily detached from the parent plant and transported along watercourses.

The hybrid has been confirmed using molecular methods from the two Chippenham specimens, which most likely represent a single genet. Two herbarium specimens have also been determined on the basis of morphology (A.C. Leslie, personal observation). One specimen is from Upware, Cambridgeshire. The other is from East Lothian, Scotland, which almost certainly will have arisen independently of the Cambridgeshire specimens. Unfortunately, we were unable to obtain permission to destructively sample these specimens, and so it has not been possible for us to verify our morphological determination using molecular methods.

The occurrence of this hybrid at more than one location, its perennial habit and its ability to spread asexually has convinced us that a hybrid binomial is warranted. There are also no intergeneric hybrids in the flora of the British Isles that do not have a hybrid generic name, and as far as we are aware this is equally true of the rest of Western Europe. Furthermore, not having a hybrid binomial would considerably hamper the future presentation of data in the frequent listings and analyses that will appear about the British flora in the coming years. The fact that the hybrid is an apparently rare, sterile clone, which propagates vegetatively is irrelevant, and is surely true of almost all intergeneric
hybrids, *e.g.* *Crataemespilus* E.G. Camus (Rosaceae), *x* *Festulpia* Melderis ex Stace & R. Cotton (Poaceae), *x* *Gaulnettya* W.J. Marchant (Ericaceae) and *x* *Tripleurothemis* Stace (Asteraceae) (Stace, 2010).
x Beruladium A.C. Leslie hyb. gen. nov. (*Berula* Besser ex W.D.J. Koch x *Helosciadium* W.D.J. Koch)

x Beruladium procurrens A.C. Leslie, hyb. nov. (*Berula erecta* (Huds.) Coville x *Helosciadium nodiflorum* (L.) W.D.J. Koch)

Holotype: England, Cambridgeshire, 26 July 2014, A.C. Leslie s.n., CGE. A large far-creeping patch, spreading from a ditch onto a very wet area of mown fen, east corner of compartment 5 (5MR), Chippenham Fen NNR (Fig. 8.5).

Diagnosis: Hybrida inter *Berulam erectam* et *Helosciadium nodiflorum*, ab illa signo annuliformi in petiolis nullo, foliis foliolorum paria modo usque ad quinque habentibus, sub umbella bracteis minus dentatis, ab hoc sub umbella bracteis plerumque aliquot, pedunculis aliquot radios aequantibus vel excedentibus differens; ab ambobus polline irregulari, fructu maturo deficiente differt.

Description: Perennial, creeping herb, spreading by numerous, prostrate, rhizomes or stolons, and readily rooting at most nodes. At the end of the season the lateral shoots on the flowering stems develop condensed leafy growths which start rooting whilst still attached and are readily detached from the parent plant. The leaves of basal vegetative shoots and lower stem leaves have up to 5 pairs of ovate to broadly ovate leaflets, the basal pair being sometimes noticeably smaller than the pair above and separated from them by a greater distance than between the second and third pair, sometimes slightly deflexed, usually unevenly crenate and sometimes shallowly lobed on the distal side. Mid and upper stem leaves with 1-3(-4) pairs of leaflets which are lanceolate to broadly ovate, unevenly crenate to serrate, but teeth not noticeably acuminate. Petioles of all leaves lack the petiolar ring characteristic of *Berula*. Flowering stems prostrate to ascending, the
prostrate ones in particular have numerous lateral shoots, which can become elongated
and entangled, with numerous lateral umbels, uppermost umbels sometimes very small.
Peduncles varying from very short to longer than rays; rays (1-)3-6(-9), slender,
subtended by (1-)2-5 bracts which are lanceolate to narrowly elliptic, usually much
shorter than the rays and entire, but rarely toothed or with a leafy tip. Sepals present but
inconspicuous. Petals white, with an acute, incurved apex. Pollen highly irregular.
Stylopodium broadly cushion-like to slightly conical. Combined length of stigma and
style is relatively long (c. 0.99-1.14 mm) when viewed in incipient fruits. The plants do
not appear to develop mature fruits, but immature fruits are ellipsoid, often with well-
developed ridges. Flowering period is prolonged, July to November.

*Berula erecta* differs from the hybrid in having much taller, erect flowering stems.
The lower leaves have 5-10(-14) pairs of leaflets and the upper stem leaves also often
have more pairs of leaflets than the hybrid. The leaflets of the mid and upper leaves in
particular are much more deeply toothed, with the teeth often acminate. All but the
smallest leaves usually exhibit a distinct ring on the petiole (formed by a membrane across
the otherwise hollow petiole), which sometimes has small leaflets or other leaf tissue
developed from it. The umbels have (5-)6-18 rays and are subtended by (3-)4-7 bracts,
which are often acuminately toothed or lobed. The stylopodium is conical and the mature
fruits are globose, with slender ridges.

*Helosciadium nodiflorum* is much more similar to the hybrid in overall
appearance, but differs from it in having 3-7 pairs of leaflets on the larger basal leaves,
the lowest pair not usually noticeably smaller than the pair above (and indeed some times
larger); all the leaf teeth are usually crenate or rather coarsely serrate. The umbels may
be sessile, and if present the peduncles are shorter than the rays, which vary in number 2-
15, and are subtended by 0-1(-2) bracts; the bracts when present are often long and almost linear, occasionally leafy at the apex. Sepals are absent or vestigial. The stylopodium is broadly cushion-shaped and the mature fruits are ellipsoid, with very prominent ridges. The combined style and stigma lengths (visible in incipient fruits) are noticeably shorter in *H. nodiflorum* (c. 0.35-0.5(-0.6) mm) than in the hybrid (c. 0.99-1.14 mm).

Etymology: *procurrens* refers to the spreading habit.

Other specimens:


Scotland, East Lothian, Haddington, Luffness, 6 August 1910, *M. Cowan Jr. s.n.*, CGE. Originally labelled as *Apium nodiflorum* var., and the subject of a long note from a variety of correspondents in the Botanical Exchange Club Report for 1910 where it was ascribed to a range of different variants of *A. nodiflorum*; determined as *A. nodiflorum* var. *ochreatum* by P.D. Sell in 2001.
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S.D. Desjardins received financial support from Natural England. S.D. Desjardins, T. Schwarzacher and J.P. Bailey are thankful to Prof. Pat Heslop-Harrison and the rest of the Molecular Cytogenetics Lab at the University of Leicester (http://molcyt.com/), where all experiments were undertaken. A.C. Leslie is grateful to the staff at Chippenham Fen NNR for access to the Fen and for permission to collect material for this study, as well as to Christine Bartram for access to material in Cambridge University Herbarium (CGE). Special thanks go to Phillip Oswald for preparing the Latin diagnosis and to Tony O’Mahony for consulting on morphological characters.
Table 1. Accessions collected from Chippenham Fen NNR, and used in the current study.

<table>
<thead>
<tr>
<th>Code</th>
<th>Voucher specimen</th>
<th>2n</th>
<th>GenBank Accession(s)</th>
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<td>7PR</td>
<td><em>Berula erecta</em> (Huds.) Coville, England, Cambridgeshire, Chippenham Fen NNR, GR 52/6479.6954, [4 October 2014] (then cultivated), <em>S.D. Desjardins &amp; A.C. Leslie</em> 1 LTR.</td>
<td>18</td>
<td>KP871508; KP871504</td>
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<tr>
<td>10MSHED</td>
<td><em>Helosciadium nodiflorum</em> (L.) W.D.J. Koch, England, Cambridgeshire, Chippenham Fen NNR, GR 52/6506.6918, [4 October 2014] (then cultivated), <em>S.D. Desjardins &amp; A.C. Leslie</em> 2 LTR.</td>
<td>22</td>
<td>KP871514; KP871507</td>
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<tr>
<td>5MR</td>
<td>x <em>Beruladium procurrens</em> A.C. Leslie (<em>B. erecta</em> x <em>H. nodiflorum</em>), England, Cambridgeshire, Chippenham Fen NNR, GR 52/6527.6936, [4 October 2014] (then cultivated), <em>S.D. Desjardins &amp; A.C. Leslie</em> 3 LTR.</td>
<td>20</td>
<td>KP871509; KP871510; KP871505</td>
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<td>10MR</td>
<td>x <em>Beruladium procurrens</em> A.C. Leslie (<em>B. erecta</em> x <em>H. nodiflorum</em>), England, Cambridgeshire, Chippenham Fen NNR, GR 52/6510.6919, [4 October 2014] (then cultivated), <em>S.D. Desjardins &amp; A.C. Leslie</em> 4 LTR.</td>
<td>20</td>
<td>KP871511; KP871512; KP871506</td>
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Figure 1. A 50% majority-rule tree (258 steps) generated by maximum parsimony analysis of rps16-trnK sequence data. The sequences from two putative Berula x Helosciadium hybrids (5MR; 10MR) were placed in a Berula clade (99% BS). BS values are displayed above nodes.
Figure 2. A 50% majority-rule consensus tree of the 24 shortest trees (437 steps) generated by maximum parsimony analysis of ITS sequence data. Two copies were detected in two putative Berula x Helosciadium hybrids (5MR; 10MR), copy 1 was placed in a B. erecta clade (100% BS) and copy 2 was placed in a H. nodiflorum clade (97% BS). BS values are displayed above nodes.
Figure 3. Aceto-orcein stained root tip metaphase spreads of *B. erecta* (A; 7PR, 2n = 18), *H. nodiflorum* (B; 10MSHED, 2n = 22) and two putative *Berula x Helosciadium* hybrids (C; 5MR, 2n = 20 & D; 10MR, 2n = 20). Scale bar = 5 μm.
Figure 4. Root tip mitotic preparations of a putative *Berula* x *Helosciadium* hybrid (10MR; 2n = 20) after genomic *in situ* hybridisation. **A+D)** Counterstained with DAPI (blue). **B--C)** Probed with total genomic DNA from *B. erecta*, labelled with biotin (red), and *H. nodiflorum*, labelled with digoxigenin (green). **E--F)** Probed with total genomic DNA from *B. erecta*, labelled with digoxigenin (green), and *H. nodiflorum*, labelled with biotin (red). At metaphase (**B+E**) 11 larger chromosomes are labelled with *H. nodiflorum* and 9 smaller chromosomes with *B. erecta*. At interphase (**C+F**) strongly fluorescent chromocentres are visible, covering larger domains in the case of *H. nodiflorum*. Scale bar = 5 μm.
Figure 5. The holotype of *Beruladium procurrens* A.C. Leslie (*Berula erecta* x *Helosciadium nodiflorum*). Scale bar = 10 cm. Courtesy of Cambridge University Herbarium (CGE).
LITERATURE CITED


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: 735--748.


## APPENDIX 1

### Supplementary information 1. Accessions used in the current study and their associated metadata. Sequences generated in the current study are indicated with an asterisk.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Accession code</th>
<th>Country of origin</th>
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<th>nrDNA GenBank accession number</th>
<th>cpDNA GenBank accession number</th>
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<td>Saint Helena</td>
<td>V. Williams 1 (WA)</td>
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<td>Saint Helena</td>
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<td>Coville – 7PR</td>
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<td>England</td>
<td>S.D. Desjardins &amp; A.C. Leslie 1 (LTR)</td>
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<td>cpDNA KP871504* – 150 ; Germany; Downie 150 (ILL); nrDNA U79607; cpDNA EF185209. Berula imbricata (Schinz) Spalik &amp; S.R. Downie</td>
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<td>Helosciadium bermejoi (L. Llorens) Popper &amp; M.F. Watson</td>
<td>62449; Balearic Islands, Spain; RGBE 19962449 (E); nrDNA AY353979; cpDNA n/a. Helosciadium crassipes W.D.J. Koch ex Rchb.; K170; France; Reduron s.n. (ILL); nrDNA AY360239; cpDNA EF185222. Helosciadium inundatum (L.) W.D.J. Koch; 64358; Sicily, Italy; Davis &amp; Sutton 64358 (E); nrDNA AF164822; cpDNA n/a. Helosciadium nodiflorum (L.) W.D.J. Koch – 10MSHED; England; S.D. Desjardins &amp; A.C. Leslie 2 (LTR); nrDNA KP871514*; cpDNA KP871507* – 317; France; Downie 317 (ILL); nrDNA EF177709; cpDNA EF185223. Helosciadium repens (Jacq.) W.D.J. Koch; 1870; France; Reduron s.n. (ILL); nrDNA AY360241; cpDNA EF367706. Sium latifolium L.; 2256; Denmark; Petersen &amp; Seberg GPL (C); nrDNA AY360258; cpDNA EF185267. Sium medium Fisch. &amp; C.A. Mey.; 2809; Kyrgyzstan; Konnov &amp; Kotshgareva 456 (LE); nrDNA DQ005674; cpDNA EF185268. Sium suave Walter; 12; Canada; Downie 12 (ILL); nrDNA AY360263; cpDNA EF185274. OUTGROUP: Hedera helix L. – 5PV97; Spain; R. Vargas 5PV97 (LIV); nrDNA AJ131227; cpDNA n/a. – 2743; n/a; Chase 2743 (K); nrDNA n/a; cpDNA GQ983991.</td>
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