Isolation and Characterization of Microsatellite Loci in Sorbus porrigentiformis and Cross-Amplification in S. aria and S. rupicola (Rosaceae)

Authors: Piñeiro, Rosalía, Karrman-Bailey, Freja, Cowan, Robyn S., and Fay, Michael F.

Source: Applications in Plant Sciences, 5(2)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1600150

Applications in Plant Sciences 2017 5(2): 1600150

Primer Note

Isolation and characterization of microsatellite loci in *Sorbus porrigentiformis* and cross-amplification in *S. aria* and *S. rupicola* (Rosaceae)[1]

Rosalía Piñeiro[2,4], Freja Karrman-Bailey[2], Robyn S. Cowan[2], and Michael F. Fay[2,3]

[2]Jodrell Laboratory, Royal Botanic Gardens, Kew, TW9 3DS Richmond, Surrey, United Kingdom; and [3]School of Plant Biology, University of Western Australia, Crawley, Western Australia 6009, Australia

- *Premise of the study:* Southwestern Britain is an emblematic hotspot of polyploid diversity of whitebeams (*Sorbus aria* agg.; Rosaceae) with ca. 30 polyploid endemic species. The tetraploid *S. porrigentiformis* is postulated as one of the parents of most of these endemics, along with the sexual diploid *S. aria* s. str. and the tetraploid *S. rupicola*.
- *Methods and Results:* We isolated 16 nuclear microsatellite loci from *S. porrigentiformis* and characterized them on 45 trees representing the three putative parental species. Eleven loci were polymorphic, and eight of them exhibited species-specific alleles. Allele numbers ranged from one to 11, and observed heterozygosity ranged from 0.40 to 1.00. The intraspecific levels of variation were very low, in agreement with the facultative apomictic reproduction hypothesized for this species.
- *Conclusions:* The species-specific alleles will be useful for tracing the origin of the narrowly distributed *Sorbus* taxa. In addition, the assessment of diversity levels will help design a conservation strategy for the polyploid complex.

Key words: British whitebeams; conservation; nuclear microsatellites; polyploid evolution; Rosaceae; *Sorbus porrigentiformis*.

British whitebeams (*Sorbus aria* aggr.; Rosaceae) are an emblematic case study of polyploid evolution in natural tree populations. Southwestern Britain is a "*Sorbus* hotspot," with ca. 30 polyploid species (3*x*, 4*x*, and even 5*x*), many of them occurring at just a few localities and therefore highly valuable in regard to conservation. Recent studies have provided detailed knowledge of the morphology and ploidy levels in British populations of *Sorbus* L. (Rich et al., 2010; Pellicer et al., 2012). Despite this effort, some essential questions regarding the evolution of the complex remain unsolved. In addition to the sexual diploid species *S. aria* (L.) Crantz s. str., current evidence points at the polyploid *S. porrigentiformis* E. F. Warb., an endemic to the United Kingdom that shows a distribution significantly larger than the other highly endemic polyploids of the complex, as a parental species of many of these polyploid endemics. The apomictic tetraploid *S. rupicola* (Syme) Hedl., widely distributed in northwestern Europe, including the United Kingdom, may have also been involved. To provide diagnostic alleles for the three species, we isolated and characterized the first set of microsatellites for *S. porrigentiformis* and tested cross-amplification in *S. aria* and *S. rupicola.* Previous studies on *Sorbus* in southwestern Britain have used two nuclear microsatellites from apple (*Malus* Mill. sp.) and three from *S. torminalis* (L.) Crantz (Robertson et al., 2010; Ludwig et al., 2013).

METHODS AND RESULTS

A DNA library was generated for one sample of *S. porrigentiformis* and sequenced on a Roche/454 GS FLX platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). From the 35,638 reads, 10,872 microsatellite loci were detected. Primer pairs were designed with the software QDD (Meglécz et al., 2010) using default parameters (90–320 bp PCR products, with more than five repeats of 2–6 bp motifs, 18–27 bp primer length, 57–63°C annealing temperature). We tested 20 of the primers on seven geographically separated individuals of *S. porrigentiformis* (Appendix 1). Fluorescent labeling was performed using three primers per locus: a reverse primer, a forward primer with a universal linker sequence (M13) at the 5′ end, and a third primer consisting of the same universal M13 sequence, labeled with 6-FAM or JOE (Schuelke, 2000). We added 7.5 µL of Multiplex Mix (10×), 0.2 µL of bovine serum albumin (BSA), 0.3 µL of each reverse primer (10 µM), 0.15 µL of dye-labeled and forward primers (10 µM), 1 µL of template DNA (ca. 10–50 ng/µL), and H_2O up to a final volume of 15 µL. Amplifications were performed as follows: 94°C (4 min); 25 or 30 cycles of 94°C (30 s), 55°C (45 s), 72°C (1 min); followed by 10 cycles each of 94°C (30 s), 53°C (45 s), 72°C (45 s); and a final extension at 60°C for 30 min. PCR products (0.7 µL) were separated on an ABI 3730 sequencer (Applied Biosystems, Lennik, The Netherlands) with 10 µL of HiDi Formamide and 0.15 µL of GeneScan 500 ROX Size Standard (Applied Biosystems). Sixteen primer combinations exhibiting robust amplification were selected (Table 1). All DNA extractions were performed with the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA).

We set up 12 simplex reactions containing one microsatellite marker and four multiplex reactions containing up to three loci (Table 1). Markers with different amplicon sizes and similar annealing temperature were identified with Multiplex Manager (Holleley and Geerts, 2009) and combined in the same multiplex. Electropherograms were automatically scored with GeneMapper version 3.7 (Applied Biosystems) and manually corrected. Fifteen markers displayed easily interpretable electropherograms with up to two alleles per locus in the diploid

[1]Manuscript received 23 November 2016; revision accepted 31 December 2016.

The authors are grateful to Timothy Fulcher for assistance with primer design and analyses. This project was funded in part by The Joseph Jones and Daisy and Graham Rattenbury Charitable Trust and by Natural England.

[4]Author for correspondence: rosalia.pineiro@gmail.com

doi:10.3732/apps.1600150

Applications in Plant Sciences 2017 5(2): 1600150
doi:10.3732/apps.1600150

TABLE 1. Description of 16 newly developed microsatellite loci in *Sorbus porrigentiformis* in four multiplex and seven simplex reactions.

	Locus[a]	Primer sequences (5′–3′)		Fluorescent label[b]	Repeat motif	Min.	Max.	*A*	GenBank accession no.
Multiplex									
PMB	SP22	F: TGATCACTTCTTACCTGCTTGG	R: CGTCATGCAGGCATATCAAT	M13-FAM	$(AC)_{14}$	234	292	11	KX090362
	SP28	F: CCATGTGCTACACCAATGGA	R: CGCACAGCAGTATAGTATATGTATCA	M13-FAM	$(ATGC)_5$	107	118	2	KX090363
	SP38	F: AAGTGAGGCGTTCCTGAGAA	R: AAGTTGAGACGAGAGTGTCGC	M13-FAM	$(AG)_{11}$	169	171	2	KX090364
PMA2	SP30	F: TACTGCTCTTCTTCCCGAGC	R: CACGACTTGGTTCCGCTAAG	M13-FAM	$(AAAGAT)_5$	203	214	3	KX090365
	SP37	F: GATGCTCGTTGCACTCTTCA	R: GAGATTCAAGGGACGAGCAG	M13-FAM	$(ACCAGC)_5$	131	156	5	KX090366
PMJ2	SP33	F: GCATCTATCCTCCATGCTACC	R: AGAAAGGAGAGTTGCAGATTTGA	M13-FAM	$(AAT)_{13}$	143	163	5	KX090367
	SP39	F: CCGTTATGGGCTATAAGGTCAG	R: TTTCTCCATTCTAGTCTGCCTC	M13-FAM	$(AT)_{13}$	271	283	6	KX090368
PMJ2	SP35	F: GTGCTTCGATAGGCGCTAGT	R: CCGGTATGAGTAGCGATTCC	M13-FAM	$(ATC)_7$	311	314	2	KX090369
	SP36	F: GTGCCAATAACAACACGCTG	R: GCCTCACTTAACCTCCTGAATG	M13-FAM	$(AT)_9$	110	147	7	KX090370
Simplex	SP29	F: AATCAAGCACACGTCTGTGAG	R: ATGGAGAAGAACTGATATAGATTAGGA	M13-JOE	$(AT)_6$	158	169	3	KX090371
	SP20	F: TTGGACGATTTCACCCAGAT	R: CTGCTCAATGATTTGTCTGGA	M13-FAM	$(AT)_{10}$	125	170	11	KX090372
	SP21	F: CATCTGCCAATTTCGTCTCCA	R: TAAGGTCTCGTCGGTTAGGG	M13-FAM	$(TTC)_5$	175	175	1	KY224065
	SP24	F: CTACTTCGGCCGTAACGATTC	R: TGTCATTGTGTTTCCCTCCC	M13-FAM	$(GGA)_5$	124	124	1	KY224066
	SP25	F: CGGGAACTCTCAACCAGACGA	R: GTTGCAAACAGGAGCTTACG	M13-FAM	$(TCA)_5$	139	139	1	KY224067
	SP26	F: CAAGAAACGGCCTGCATAGAC	R: AGAGAACCCGTTCGTTGTGT	M13-FAM	$(CAG)_6$	239	245	2	KY224068
	SP34	F: CTCCAGAGGAGGAAGTGAAAGA	R: AATTTCAATGGTGTCGGTCC	M13-FAM	$(AGC)_5$	189	189	1	KY224069

Note: *A* = number of alleles; Max. = maximum allele size; Min. = minimum allele size.
[a]The annealing temperature was 55°C for all loci.
[b]M13 = CACGACGTTGTAAAACGAC (Schuelke, 2000).

TABLE 2. Genetic diversity of the 11 newly developed polymorphic microsatellites in three populations of *Sorbus porrigentiformis* and cross-amplification in *S. aria* and *S. rupicola*. All populations are located in southwestern Britain.

		S. porrigentiformis (4*x*, 3*x*) (*N* = 25)											*S. rupicola* (4*x*) (*N* = 10)					*S. aria* (2*x*) (*N* = 10)				
				Bristol (*N* = 5)			Somerset (*N* = 9)			Wales (*N* = 11)												
	Locus	Private	A_p	H_o	H_e	$H_{e\text{-}d}$	H_o	H_e	$H_{e\text{-}d}$	H_o	H_e	$H_{e\text{-}d}$	Min.	Max.	*A*	Private	A_p	Min.	Max.	*A*	Private	A_p
Multiplex																						
PMB	SP22	245, 255, 257, 263, 270, 284, 292	7	—	—	—	—	—	—	—	—	—	234	276	5	249, 264, 276	3	234	268	4	256	1
	SP28			1.00	0.56	0.55	1.00	0.53	0.53	0.91	0.52	0.51	107	118	2			107	118	2		
	SP38	171	1	1.00	0.56	0.51	0.78	0.53	0.48	0.91	0.52	0.51	165	165	1			165	169	3	166	1
PMA2	SP30			0.40	0.48	0.62	0.89	0.63	0.60	1.00	0.57	0.54	208	214	2			203	214	3		
	SP37	131	1	1.00	0.71	0.75	1.00	0.74	0.73	1.00	0.69	0.69	137	150	2			137	156	4		
PMJ2	SP33	156	1	1.00	0.56	0.79	1.00	0.78	0.72	1.00	0.57	0.55	143	150	2			140	163	6	140, 160	2
	SP39	277, 283	2	1.00	0.79	0.79	1.00	0.80	0.80	1.00	0.77	0.77	271	285	4	285	1	271	310	5	292, 310	2
PMJ2	SP35			1.00	0.56	0.55	1.00	0.53	0.53	0.91	0.52	0.51	311	314	2			311	311	1		
	SP36	132, 138, 140	3	1.00	0.75	0.86	1.00	0.83	0.81	1.00	0.71	0.70	143	157	6	150, 152, 154, 157	4	110	147	5	143	1
Simplex																						
	SP29	166	1	1.00	0.56	0.68	0.88	0.63	0.50	0.90	0.53	0.51	154	158	3	156	1	154	169	3	169	1
	SP20	125, 132, 149, 152, 154, 156	6	1.00	0.82	0.83	1.00	0.82	0.78	1.00	0.74	0.73	140	174	5	140, 172, 174	3	123	170	5	123	1

Note: *A* = number of alleles; A_p = number of private alleles; H_e = expected heterozygosity; $H_{e\text{-}d}$ = expected heterozygosity corrected by allele dosages; H_o = observed heterozygosity; Max. = maximum allele size; Min. = minimum allele size; *N* = number of individuals sampled; Private = size of private alleles.

individuals and up to four alleles in the tetraploid individuals. Locus SP22 exhibited up to four peaks in diploids and up to six in tetraploids. Two different size ranges with different amplification intensities and up to two peaks per individual each could be distinguished in *S. aria* and *S. rupicola*, but not in *S. porrigentiformis*. Therefore, locus SP22 was analyzed as a dominant marker.

To characterize the 16 microsatellite loci, 45 individuals were genotyped (Appendix 1): 25 *S. porrigentiformis* from three different populations in southwestern Britain (3*x* and 4*x*), 10 *S. aria* (2*x*), and 10 *S. rupicola* (4*x*). *Sorbus porrigentiformis* is endemic to southwestern Britain and individuals occur scattered in the field, which explains the limited sample sizes in this study. However, given that reproduction is mostly clonal, our sampling strategy is representative of the real genetic variation of the species. Ploidy levels of all samples were known from a previous flow cytometry study (Pellicer et al., 2012). Five markers were monomorphic across all 45 samples studied (Table 1). Locus SP26 was biallelic, whereas SP21, SP24, SP25, and SP34 were monoallelic. The remaining 11 microsatellite markers were polymorphic across the three congeners (Tables 1, 2), eight of them exhibited species-specific alleles. Twenty-two private alleles were identified for *S. porrigentiformis*. Locus SP28, although monomorphic in terms of allele counts, exhibited species-specific differences in allele dosage between *S. porrigentiformis* and *S. rupicola* that could be clearly detected, with a ratio of peak areas of 0.45 and 1.34, respectively (Esselink et al., 2004).

For the 11 polymorphic loci, one to 11, one to six, and one to six alleles per locus were retrieved for *S. porrigentiformis*, *S. aria*, and *S. rupicola*, respectively (Tables 1, 2). Allele sizes, number of alleles, and number of private alleles were calculated for each polymorphic locus and species using SPAGeDi (Hardy and Vekemans, 2002). *Sorbus porrigentiformis* genotypes were further evaluated with GENODIVE (Meirmans and Van Tienderen, 2004) by estimating the expected and observed heterozygosity, with and without correction of allele dosages for polyploids using a maximum likelihood method. Within *S. porrigentiformis*, populations for most loci exhibited fixed alleles. The observed heterozygosity varied between 0.40 and 1.00. *Sorbus porrigentiformis* exhibited low genetic variation at the intraspecific level, but it was not completely clonal, fitting the expectations for a facultative apomict.

CONCLUSIONS

The newly developed nuclear microsatellite loci allow discrimination between the species *S. porrigentiformis*, *S. aria*, and *S. rupicola*. These markers will be an important tool to trace the origin of polyploid endemic species of the *S. aria* agg. in southwestern Britain, and to understand the relative contribution of *S. aria*, *S. rupicola*, and *S. porrigentiformis* as parents of these local polyploids. The resulting genetic information will be relevant for choosing the best approach for the conservation of the polyploid complex *S. aria* agg. in southwestern Britain either by focusing on the conservation of the local endemic taxa or by focusing on the preservation of the polyploidization process (Ennos et al., 2012) by protecting the parental species, even if they are not local endemics themselves.

LITERATURE CITED

Ennos, R. A., R. Whitlock, M. F. Fay, B. Jones, L. E. Neaves, R. Payne, I. Taylor, et al. 2012. Process-based Species Action Plans: An approach to conserve contemporary evolutionary processes that sustain diversity in taxonomically complex groups. *Botanical Journal of the Linnean Society* 168: 194–203.

Esselink, G. D., H. Nybom, and B. Vosman. 2004. Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting–peak ratios) method. *Theoretical and Applied Genetics* 109: 402–408.

Hardy, O. J., and X. Vekemans. 2002. SPAGeDi: A versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* 2: 618–620.

Holleley, C. E., and P. G. Geerts. 2009. Multiplex Manager 1.0: A cross-platform computer program that plans and optimizes multiplex PCR. *BioTechniques* 46: 511–517.

Ludwig, S., A. Robertson, T. C. G. Rich, M. Djordjević, R. Cerović, L. Houston, S. A. Harris, and S. J. Hiscock. 2013. Breeding systems, hybridization and continuing evolution in Avon Gorge *Sorbus*. *Annals of Botany* 111: 563–575.

Meglécz, E., C. Costedoat, V. Dubut, A. Gilles, T. Malausa, N. Pech, and J.-F. Martin. 2010. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics (Oxford, England)* 26: 403–404.

Meirmans, P. G., and P. H. Van Tienderen. 2004. GENOTYPE and GENODIVE: Two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 4: 792–794.

Pellicer, J., S. Clermont, L. Houston, T. C. G. Rich, and M. F. Fay. 2012. Cytotype diversity in the *Sorbus* complex (Rosaceae) in Britain: Sorting out the puzzle. *Annals of Botany* 110: 1185–1193.

Rich, T. C. G., L. Houston, A. Robertson, and M. Proctor. 2010. Whitebeams, rowans and service trees of Britain and Ireland: A monograph of *Sorbus* L. in Britain and Ireland. Botanical Society of Britain and Ireland, Shirehampton, Bristol, United Kingdom.

Robertson, A., T. C. G. Rich, A. M. Allen, L. Houston, C. A. T. Roberts, J. R. Bridle, S. A. Harris, and S. J. Hiscock. 2010. Hybridization and polyploidy as drivers of continuing evolution and speciation in *Sorbus*. *Molecular Ecology* 19: 1675–1690.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

Appendix 1. Voucher information for *Sorbus* populations characterized in this study. All collections are located in southwestern Britain. Herbarium vouchers are deposited in the Welsh National Herbarium (NMW).

Voucher no.	Species	Ploidy level	Collection locality	Collection date[a]	Collector	Latitude (°N)	Longitude (°E)
L139	*S. aria*	2*x*	Burrington Combe	15/08/11	L. Houston	51.32	−2.74
L106	*S. aria*	2*x*	Cheddar Gorge S side	09/08/11	L. Houston	51.29	−2.75
FC066	*S. aria*	2*x*	East Wood, Portishead	11/07/11	L. Houston, M. Fay, J. P. Moscardo, S. Clermont, T. Rich	51.49	−2.77
FC067	*S. aria*	2*x*	East Wood, Portishead	11/07/11	L. Houston, M. Fay, J. P. Moscardo, S. Clermont, T. Rich	51.49	−2.77
FC353	*S. aria*	2*x*	Gorrashill Wood	11/08/11	T. Rich	51.64	−2.71
FC018	*S. aria*	2*x*	Leigh Woods, Quarry 4	04/07/11	T. C. G. Rich, L. Houston, S. Ludwig, I. Trotman	51.46	−2.63
FC156	*S. aria*	2*x*	Offa's Dyke, Tidenham Chase	12/07/11	M. Fay, J. P. Moscardo, S. Clermont, T. Rich	51.68	−2.66
FC109	*S. aria*	2*x*	Seven Sisters	12/07/11	M. Fay, J. P. Moscardo, S. Clermont, T. Rich	51.83	−2.66
L130	*S. aria*	2*x*	Weston Big Wood: Valley Road	15/08/11	L. Houston	51.47	−2.79
FC056	*S. aria*	2*x*	Worlebury Hill, west end	11/07/11	L. Houston, M. Fay, J. P. Moscardo, S. Clermont, T. Rich	51.36	−2.99
FC168	*S. rupicola*	4*x*	Craig y Cilau NNR	18/07/11	T. C. G. Rich	51.83	−3.17
FC173	*S. rupicola*	4*x*	Craig y Cilau NNR	18/07/11	T. C. G. Rich	51.83	−3.18
FC174	*S. rupicola*	4*x*	Craig y Cilau NNR	18/07/11	T. C. G. Rich	51.83	−3.18
FC315	*S. rupicola*	4*x*	Neck Wood, Trentishoe	10/08/11	T. Rich & S. Whild	51.22	−3.96
FC320	*S. rupicola*	4*x*	Neck Wood, Trentishoe	10/08/11	T. Rich & S. Whild	51.22	−3.96
FC197	*S. rupicola*	4*x*	Penmoelallt	18/07/11	T. C. G. Rich	51.77	−3.43
FC203	*S. rupicola*	4*x*	Penmoelallt	18/07/11	T. C. G. Rich	51.77	−3.43
FC205	*S. rupicola*	4*x*	Penmoelallt	18/07/11	T. C. G. Rich	51.77	−3.43
FC102	*S. rupicola*	4*x*	Seven Sisters	12/07/11	M. Fay, J. P. Moscardo, S. Clermont, T. Rich	51.83	−2.66
FC106	*S. rupicola*	4*x*	Seven Sisters	12/07/11	M. Fay, J. P. Moscardo, S. Clermont, T. Rich	51.83	−2.66
FC306	*S. porrigentiformis* s. str.	4*x*	Bristol	28/08/11	T. C. G. Rich	—	—
L039	*S. porrigentiformis* s. str.	4*x*	Bristol	23/07/11	L. Houston	51.47	−2.64
L041	*S. porrigentiformis* s. str.	4*x*	Bristol	23/07/11	L. Houston	51.47	−2.64
L044	*S. porrigentiformis* s. str.	4*x*	Bristol	23/07/11	L. Houston	51.46	−2.63
L124	*S. porrigentiformis* agg.	4*x*	Bristol	15/08/11	L. Houston	51.50	−2.64
L046	*S. porrigentiformis* s. str.	4*x*	Somerset	25/07/11	L. Houston	51.28	−2.77
L050	*S. porrigentiformis* s. str.	4*x*	Somerset	25/07/11	L. Houston	51.28	−2.77
L069	*S. porrigentiformis* s. str.	4*x*	Somerset	25/07/11	L. Houston	51.28	−2.76
L074	*S. porrigentiformis* s. str.	4*x*	Somerset	25/07/11	L. Houston	51.28	−2.76
L077	*S. porrigentiformis* s. str.	4*x*	Somerset	25/07/11	L. Houston	51.28	−2.76
L089	*S. porrigentiformis* s. str.	4*x*	Somerset	07/08/11	L. Houston	51.20	−2.42
L115	*S. porrigentiformis* agg.	3*x*	Somerset	10/08/11	L. Houston	51.28	−2.76
L136	*S. porrigentiformis* agg.	3*x*	Somerset	15/08/11	L. Houston	51.32	−2.74
L138	*S. porrigentiformis* agg.	3*x*	Somerset	15/08/11	L. Houston	51.32	−2.74
FC164	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.84	−3.21
FC171	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.83	−3.17
FC176	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.84	−3.18
FC185	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.84	−3.18
FC188	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.84	−3.18
FC191	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.84	−3.18
FC192	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.84	−3.18
FC194	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.84	−3.19
FC207	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.54	−3.26
FC212	*S. porrigentiformis* s. str.	4*x*	Wales	18/07/11	T. C. G. Rich	51.80	−3.08
FC301	*S. porrigentiformis* s. str.	4*x*	Wales	28/08/11	T. C. G. Rich	51.83	−3.17

Note: NNR = National Nature Reserve.

[a]Collection date is presented as day/month/year.