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Cytotype diversity in the *Sorbus* complex (Rosaceae) in Britain: sorting out the puzzle

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• *Background and Aims* Large-scale ploidy surveys using flow cytometry have become an essential tool to study plant genome dynamics and to gain insight into the mechanisms and genetic barriers framing ploidy diversity. As an ideal complement to traditional techniques such as chromosome counting, the analysis of cytotype diversity in plant systems such as *Sorbus* provides primary investigation into the potential patterns and evolutionary implications of hybrid speciation.
• *Methods* Ploidy was assessed by means of relative nuclear DNA content using propidium iodide flow cytometry in 474 *Sorbus* samples collected from 65 populations in southern Wales and South-West England. Statistical tests were applied to evaluate the utility of this technique to confidently discriminate ploidy in the genus.
• *Key Results* Flow cytometric profiles revealed the presence of four cytotypes (2*x*, 3*x*, 4*x* and 5*x*), confirming in many cases chromosome counts previously reported and demonstrating cytotype heterogeneity within specific *Sorbus* aggregates. Diploid cytotypes were restricted to the potential parental species and homoploid hybrids. Most of the samples processed were polyploid. The occurrence of the pentaploid cytotype had previously only been reported from a single specimen; it is now confirmed for two taxa occurring at different sites.
• *Conclusions* Flow cytometry results obtained have proved useful in shedding light on the taxonomy of several controversial taxa and in confirming the presence of cytoypes which occur at very low frequencies. Notably, the coexistence of several cytotypes in *Sorbus* populations has probably been facilitated by the overlapping distribution of many of the species studied, which might also explain the high incidence of potential hybrid apomictic polyploids. These results will provide a solid baseline for molecular research aiming to better understand the genetic pathways controlling the formation and establishment of polyploid *Sorbus*.

Key words: Apomixis, cytotype mixture, flow cytometry, genome size, hybridization, ploidy, polyploidy, whitebeam.

INTRODUCTION

The genus *Sorbus* in Britain is represented by 45 native and seven introduced taxa (Rich *et al.*, 2010). The origin of polyploid endemics in the genus is an ongoing question, but early studies (e.g. Liljefors, 1955) suggested that the sexual diploids *S. aria*, *S. aucuparia* and *S. torminalis*, with the apomictic tetraploid *S. rupicola*, probably form the backbone of this complex. In the last decade, new molecular markers for investigating population genetics have become increasingly useful in disentangling relationships in the genus, revealing a complex evolutionary history (Fig. 1) in which hybridization and recurrent episodes of polyploidization have played a crucial role (Nelson-Jones *et al.*, 2002; Chester *et al.*, 2007). The general interest that polyploidy has raised among researchers due to its frequent occurrence in plant systems has been essential in gaining insight into the dynamics of this evolutionary force and its implications in plant speciation (Soltis *et al.*, 2009). Despite these advances, the potential advantages of apomixis in plant diversification and colonization (Hörandl *et al.*, 2008), as well as the reproductive mechanisms of apomixis relating to polyploidy (Dickinson *et al.*, 2007), are not fully understood and are still areas of active study.

The number of species recognized in *Sorbus* varies between authors depending on the way that the numerous polyploid apomicts are treated, and the classification has been frequently revised (Proctor, 1999; Aldasoro *et al.*, 2004; Rich and Proctor, 2009). Taxa such as the so-called 'No Parking' whitebeam, now known as *S. admonitor*, and *Sorbus* 'Taxon D', now known as *S. margaretae*, were unnamed for a long time (Proctor *et al.*, 1989). Given that many of these polyploids originated via hybridization, the question arises as to whether they should be considered as hybrids or as new independent species, a question that might be related to the ability of these hybrid genotypes to produce viable offspring and perpetuate themselves. Nearly all taxa in the genus are relatively long-lived trees, mostly needing high light levels and are intolerant of grazing. For that reason many species have a preference for open cliffs where they have easy access to sunlight. As exceptions, *S. aucuparia* and representatives of the *S. eminens* aggregate are shade-tolerant, and *S. torminalis* is the only woodland species of *Sorbus* in Britain.

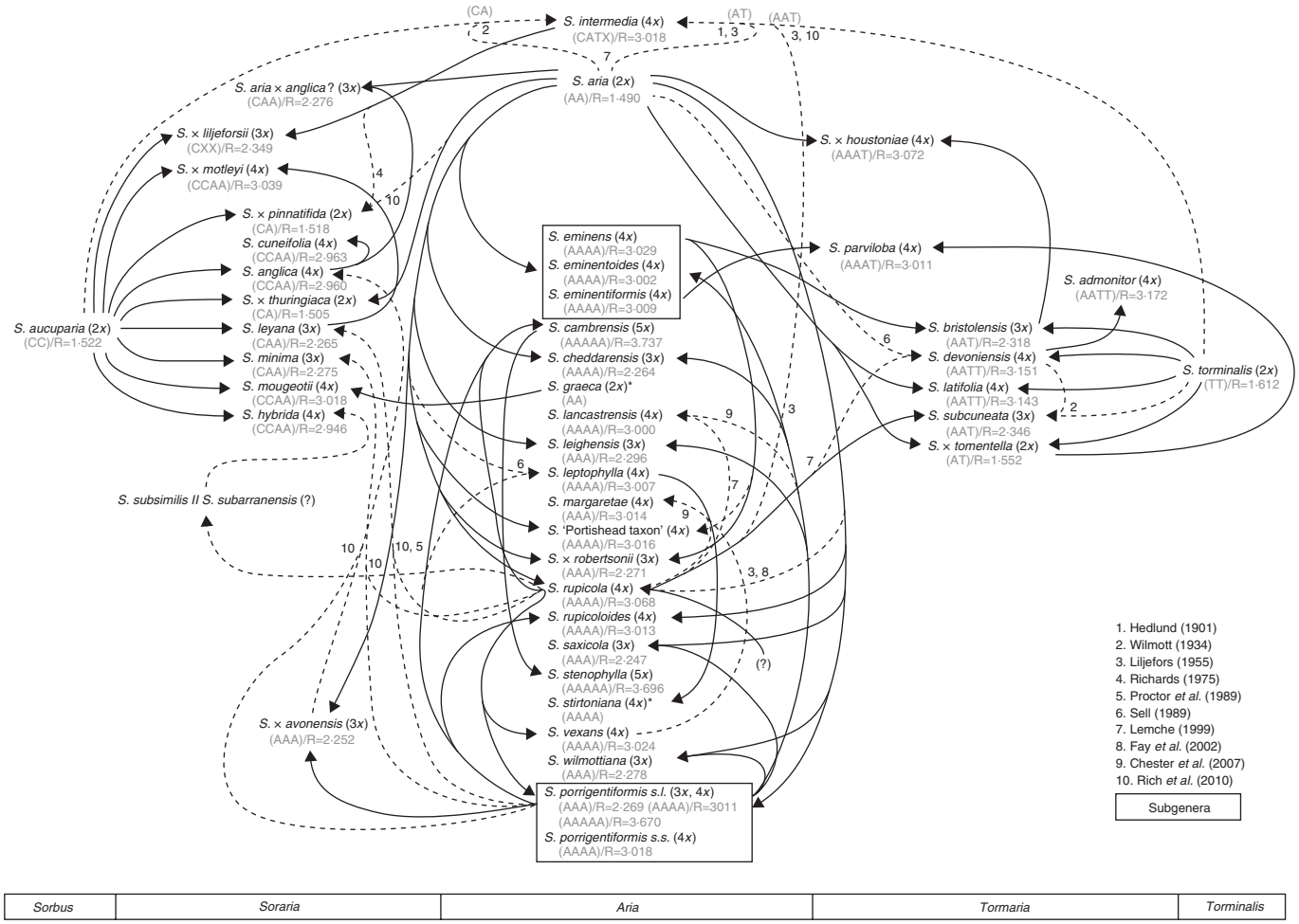

FIG. 1. Hypothesized network of relationships in *Sorbus* based on the results compiled by Rich *et al.* (2010) including diploids and the intermediate polyploid taxa. Proposed genome compositions are also included with the relative DNA content and ploidy inferred using FCM. Dotted lines indicate either multiple or dubious origins. *Ploidy based on published chromosome numbers (Warburg and Kárpáti, 1968; Bailey *et al.*, 2008).

From a karyological point of view, chromosome counts have only been reported for 24 native *Sorbus* taxa. Estimates of ploidy inferred from the number of microsatellite alleles are also available for nine taxa, but chromosome numbers in 12 of the most recently described taxa are still unknown (Bailey *et al.*, 2008; Houston *et al.*, 2009; Robertson *et al.*, 2010). Karyological information is available for all the introduced taxa. Three common ploidies (diploid, triploid and tetraploid; $2n = 34$, 51 and 68, respectively) occur in Britain, and a single 'pentaploid' count ($2n =$ approx. 87 chromosomes) was reported for a seedling of *S. porrigentiformis sensu lato* (*s.l.*) (Bailey *et al.*, 2008). Most taxa have only been reported to occur at one ploidy, but three of the native taxa have been recorded to have more than one cytotype; *S. anglica* and *S. subcuneata* have both been reported to include triploids and tetraploids, and *S. porrigentiformis s.l.* includes triploids and tetraploids. The single pentaploid count was obtained from a cultivated seedling and, in the absence of information regarding the parentage of the seed, this count might not be representative of that for the parent. The introduced *S. latifolia* has been reported as both diploid and tetraploid, although the diploid counts probably refer to *S.* × *tomentella* (the primary hybrid between *S. aria* and *S. torminalis*).

The difficulties in obtaining suitable tissue for chromosome counts and the small size and relatively high numbers of chromosomes clearly indicate a need to find alternative techniques to estimate ploidy easily. The availability of knowledge of ploidy in addition to karyological data (see compilation of Bailey *et al.*, 2008) could become a source of extra information to help in inferring species relationships and in hypothesizing the origins in controversial cases. Flow cytometry (hereafter FCM) has become widely used for estimating nuclear DNA contents in either absolute or relative (i.e. DNA ploidy) units, with many applications described in fields such as plant genome size diversity, biosystematics, ecology and population biology (Kron *et al.*, 2007; Loureiro *et al.*, 2010; Pellicer *et al.*, 2010).

Recently, understanding of the patterns of occurrence and distribution of polyploids across plant populations has been facilitated by easy access to FCM, enabling us to explore the evolutionary implications of hybrid speciation (Leitch *et al.*, 2008; Siljak-Yakovlev *et al.*, 2008; Bennert *et al.*, 2011;

Marques *et al.*, 2012). In this sense, FCM has become an ideal tool for tracking the dynamics of ploidy variation in many diverse plant groups and assessing the processes that frame cytotype distribution on a temporal scale (Ebihara *et al.*, 2005; Suda *et al.*, 2007; Marhold *et al.*, 2010; Šafářová and Duchoslav, 2010; Trávníček *et al.*, 2010, 2011). This technique is also especially useful in providing a more robust understanding of genetic data at the population level. An illustrative example can be found in the genus *Gymnadenia* (Orchidaceae). While chromosome studies in the complex (Marhold *et al.*, 2005) reported the existence of 2*x* and 4*x* cytotypes, and nuclear microsatellite data (Stark *et al.*, 2011) were useful only in differentiating between diploid and polyploid populations *sensu lato*, the ploidy screening carried out by Trávníček *et al.* (2011) using FCM revealed the coexistence of multiple cytotypes (2*x*, 3*x*, 4*x*, 5*x*, 6*x*) in the genus. Scenarios such as this not only show the importance of large-scale surveys, but also highlight the difficulties in interpreting genetic data for markers such as nuclear microsatellites for which allele number is dependent on ploidy (e.g. *Sorbus*; M. F. Fay *et al.*, unpubl. res.). This illustrates the need to explore potential cytotype coexistence under environmental conditions by using complementary tools that provide data for gaining insight into the mechanisms of polyploidy and speciation, and assessing the genetic boundaries preserving such diversity and its consequences at the genome size level.

Given the previous evidence suggesting a frequent co-occurrence of cytotypes in British *Sorbus* (Bailey *et al.*, 2008), we designed a population survey: (1) to ascertain the diversity of cytotypes in a range of species to help in clarifying species relationships; (2) to assess the coexistence of multiple ploidies within species and aggregates; and (3) to provide a general picture of the most prevalent cytotypes in British *Sorbus* populations.

MATERIALS AND METHODS

Plant material

Sixty-five populations of *Sorbus*, including 31 recognized species, seven known interspecific hybrids and 15 taxonomically unconfirmed specimens, were collected during summer 2011 covering (in many cases) the ranges of the known distribution of the rarer taxa (see Supplementary Data Table S1, available online, for locality and herbarium voucher information). In this study, the field sampling aimed to cover as wide a range of species in South-West England and Wales as possible with a good coverage of diploids to provide background data and information on known taxonomically difficult individuals. Additional collections from the National Botanic Garden of Wales, the Finnish Museum of Natural History and the Pyrenees were also included. In total, 474 samples were cytotyped. Herbarium vouchers are deposited in the Welsh National Herbarium (NMW).

Chromosome counts

Karyological information based on chromosome numbers compiled in the literature (Bailey *et al.*, 2008; Rich *et al.*, 2010) was used to confirm whether these previous reports correlated with our DNA ploidy estimations using FCM.

Flow cytometry

Samples were processed by FCM using propidium iodide (PI)-stained nuclei to unravel DNA ploidy levels (Suda *et al.*, 2006). Fully expanded leaf tissue from each specimen collected in the field was preserved in plastic bags at 4 °C for up to 5 d. About 1 cm^2 of the target sample was co-chopped with the selected internal standard (*Oryza sativa* 'IR36', 1C = 0·5 pg) using a new razor blade in a Petri dish containing 1 mL of 'general purpose isolation buffer' (GPB; Loureiro *et al.*, 2007) with 3 % PVP-40 following the one-step procedure described by Doležel *et al.* (2007). The nuclear suspension was then filtered through a nylon mesh (30 μm) to remove debris, stained with PI (1 mg mL^{-1}; Sigma) at a final concentration of 60 μg mL^{-1} and supplemented with 17 μL of a solution of 3 mg mL^{-1} ribonuclease A (RNase A; Sigma). After incubation for 10 min on ice, the relative nuclear DNA content was estimated by recording at least 3000 particles using a Partec Cyflow SL3 (Partec GmbH, Münster, Germany) flow cytometer fitted with a 100-mW green solid state laser (Cobolt Samba). The resulting histograms were analysed with the FlowMax software (v. 2·4, Partec GmbH). The absolute DNA content of the diploid species *S. aria*, *S. aucuparia* and *S. torminalis* was calculated following the same procedure described above but adjusting the acquisition to 5000 particles and analysing three independent specimens (three replicates each). Ploidy was determined on the basis of the sample/standard ratio, taking into account the FCM profiles of the diploid taxa. Given that only *Oryza sativa* was used as standard and its 2C-value is 1·0 pg, ratios calculated are equivalent to the relative DNA content.

Statistical analysis

Data were analysed with Statgraphics Plus v. 5·1 (Statistical Graphics Corp., Warrenton, VA, USA). The normality of the data distributions was tested using the Kolmogorov–Smirnov test and the homogeneity of variances by Levene's test. Differences between selected groups were tested using the non-parametric Kruskal–Wallis test. A linear regression analysis was also conducted to test for any correlation between relative DNA content and ploidy.

RESULTS

Accuracy of measurements: evaluation of flow histograms

The *Sorbus* samples analysed produced high-resolution flow histograms with peaks of both the target and standard samples yielding low coefficients of variation (CV%): 1·80–3·50 (mean = 2·39 ± 0·52) and 1·22–3·65 (mean = 2·23 ± 0·45) (Fig. 2). Little variation (<2·8 %) was found between samples from the same species measured on different days. In most cases, only peaks with nuclei in phase G_0/G_1 of mitosis (2C peak) were recorded; those for G_2 nuclei were almost imperceptible and did not interfere with interpretation

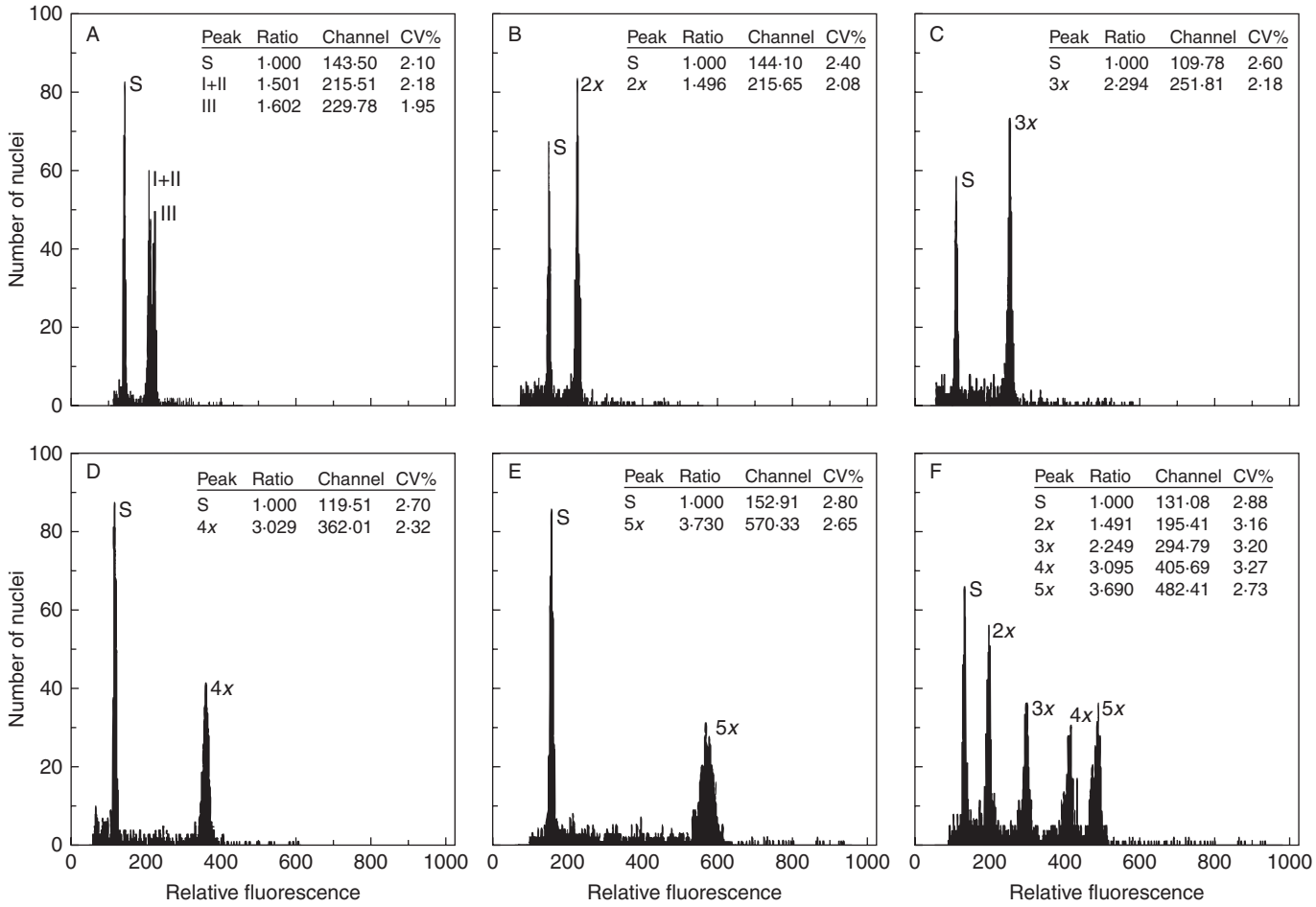

FIG. 2. Representative flow cytometric histograms illustrating all DNA ploidies found within the *Sorbus* complex investigated [internal standard *Oryza sativa* (S)]. Nuclei of both the target samples and the standard were isolated together and stained with PI. (A) combined sample of the diploid species *S. aria* (I), *S. aucuparia* (II) and *S. torminalis* (III); (B) diploid *S. aria*; (C) triploid *S. leyana*; (D) tetraploid *S. eminens*; (E) pentaploid *S. cambrensis*; (F) combined run including all cytotypes and the internal standard.

TABLE 1. *Nuclear DNA amounts estimated for the parental diploid* Sorbus

Taxon	2*n*	Ploidy	2C (pg, mean ± s.d.)	1C (pg)	1C (Mbp)*
S. aria (L.) Crantz	34	2*x*	1·484 ± 0·023	0·742	752·676
S. aucuparia (L.)	34	2*x*	1·525 ± 0·011	0·762	745·236
S. torminalis (L.) Crantz	34	2*x*	1·612 ± 0·002	0·810	792·180

* 1 pg = 978 Mbp (Doležel *et al.*, 2003).

of the results when present. The genome sizes of the diploid parental taxa were similar (Table 1, Fig. 2A), although that of *S. torminalis* was slightly larger (also reflected in the hybridogenous taxa with *S. torminalis* as one parent providing useful information on origins). Four easily recognizable distinct groups of fluorescence intensities were observed in taxa with chromosome counts available, therefore allowing us to infer reliable DNA ploidy (Fig. 2B–F).

Ploidy variation in the Sorbus *complex*

As expected, DNA peak ratios identified ploidy (Fig. 3; $K = 409{\cdot}83$, $P < 0{\cdot}0001$) based on the assumption that ratios ranging from 1·434 to 1·631 represented DNA-diploid individuals, from 2·286 to 2·370 triploids, 2·882 to 3·226 tetraploids and 3·647 to 3·833 pentaploids. Thus, four different cytotypes (2*x*, 3*x*, 4*x* and 5*x*) were found to occur among the samples analysed (Table 2, Supplementary Data Table S1). An illustrative flow cytometric histogram combining taxa of all different ploidies reported is presented in Fig. 2F.

The linear relationship between the relative DNA content and ploidy was confirmed (Supplementary Data Fig. S1; $R^2 = 0{\cdot}99$, $P < 0{\cdot}0001$). Of the samples collected, tetraploids were most frequent (41 % of the samples analysed), followed by triploids (29 %), diploids (26 %) and pentaploids (4 %), but note that we focused on individuals which we expected to be polyploids. Spatial ploidy coexistence was frequent in *Sorbus* populations, and in approx. 70 % of the sites visited at least two cytotypes were reported (see Supplementary Data Table S1). Craig y Cilau National Nature Reserve in Wales harboured all four ploidies.

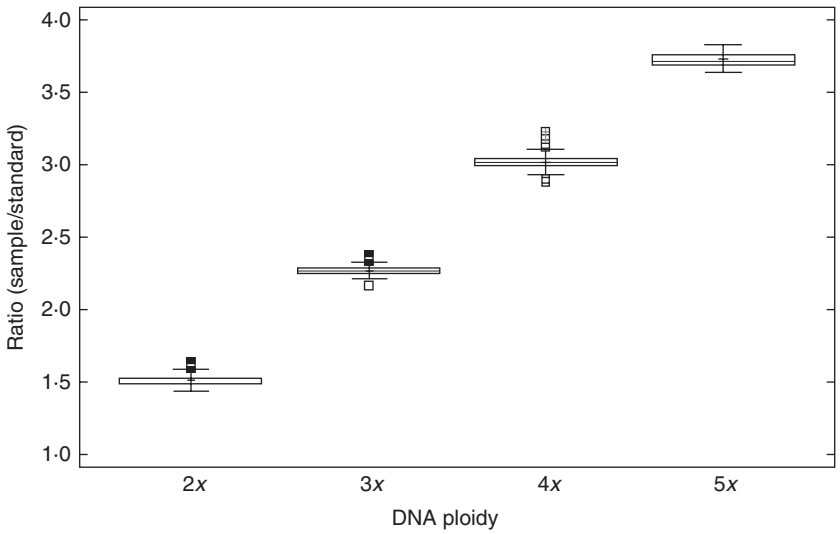

FIG. 3. Box and whisker plot representation of the relative DNA content according to ploidy in *Sorbus*.

Species cytotype identification

The results for the inferred DNA ploidies of the studied *Sorbus* taxa are summarized in Tables 2 and 3, and detailed information for each specimen assessed can be found in Supplementary Data Table S1.

Of the diploids, *S. aria* (but see below), *S. aucuparia* and *S. torminalis* were found to be constant in the sampling (see Supplementary Data Table S1), with their FCM profiles discriminating between species ($K = 57{\cdot}732$, $P < 0{\cdot}0001$; Supplementary Data Fig. S2A), most clearly in the case of *S. torminalis* (Fig. 4). The expected results were confirmed in the homoploid hybrids *S.* × *thuringiaca* (*S. aria* × *S. aucuparia*) and *S.* × *tomentella* (*S. aria* × *S. torminalis*), the relative DNA contents of which fell between those of the hypothesized parents (Figs 1 and 4, Supplementary Data Fig. S2B). However, 14 specimens from seven sites collected as dubious *S. aria* were triploid. The taxonomic utility of the FCM records at higher levels was also tested in triploids and tetraploids (Supplementary Data Fig. S2C, D). Although polyploids of subgenus *Tormaria* (Fig. 1, subgenus *Aria* × subgenus *Torminaria*) were generally discriminated (Fig. 4; 3*x*, $K = 13{\cdot}133$, $P = 0{\cdot}0013$; 4*x*, $K = 36{\cdot}682$, $P < 0{\cdot}0001$), those belonging to subgenera *Aria* and *Soraria* (subgenus *Aria* × subgenus *Sorbus*) had overlapping genome sizes due to the similar nuclear DNA content of the parental taxa and could not be distinguished (Supplementary Data Fig. S2C, D).

Of the polyploid apomictic species in subgenus *Aria*, three were inferred as triploid and ten as tetraploid cytotypes (Table 2). The hybrids *S.* × *robertsonii* (*S. aria* × *S. eminens*) and *S.* × *avonensis* (*S. aria* × *S. porrigentiformis*) were triploid as expected (Table 3), and *S. cambrensis* and *S. stenophylla* were shown to be fixed pentaploids. Different cytotypes were found in *S. parviloba*, one tetraploid (the type tree) and two triploids. Specimens collected as members of the *S. porrigentiformis* group included both triploid and tetraploid cytotypes.

In subgenus *Soraria* (members of which generally originated as subgenus *Aria* × subgenus *Sorbus*), *S. minima* and *S. leyana* were triploid. The backcross between *S. leyana* and *S. aucuparia* (= *S.* × *motleyi*) was tetraploid, as were *S. cuneifolia* and *S. mougeotii*. *Sorbus hybrida* was also tetraploid, but the sample collected as a backcross with *S. aucuparia* (of cultivated origin) was unexpectedly diploid. *Sorbus intermedia*, generally included in this subgenus [although it also has *S. torminalis* in its genome (Fig. 1; Rich *et al.*, 2010)], was tetraploid, and its backcross with *S. aucuparia* (= *S.* × *liljeforsii*) was triploid as expected. *Sorbus anglica*, previously reported as both triploid and tetraploid, was found to be consistently tetraploid. One possible *S. anglica* × *S. aria* hybrid from Cheddar was found to be triploid and will be investigated in more detail.

Results for subgenus *Tormaria* (members of which generally originated as subgenus *Aria* × subgenus *Torminaria*) were as follows: *S. bristoliensis* was confirmed to be triploid and its backcross with *S. aria* (= *S.* × *houstoniae*) tetraploid. The latter cytotype was also found in *S. admonitor*, *S. devoniensis* and *S. latifolia*. Although *S. subcuneata* had previously been reported at different ploidies (3*x*, 4*x*), the samples studied here were consistently triploid.

DISCUSSION

Efficacy of FCM for ploidy estimation in Sorbus

Regarding the utility of FCM, unlike in *Crataegus* (also Rosaceae; Talent and Dickinson, 2005), in which assessment of ploidy was sometimes difficult due to overlapping ranges of genome size, DNA ploidy inferred in *Sorbus* was highly reliable and clearly differentiated between cytotypes (Figs 3 and 4). This situation has been mainly reported in surveys restricted at either the species (Balao *et al.*, 2009; Cosendai and Hörandl, 2010) or small aggregate (Marhold *et al.*, 2010; Trávníček *et al.*, 2011) levels, but in large complexes such in *Sorbus*, where multiple hybridization episodes are frequent, it would not be surprising to have found more complex FCM profiles as a result of different evolutionary histories.

TABLE 2. *Karyological data and population cytotypes for the studied* Sorbus *taxa (for more detailed information about specimen localities and FCM results, see Supplementary Data Table S1)*

Taxon	2*n**	Ploidy	DNA ploidy†	No. of specimens	No. of populations	Ratio‡ (mean ± s.d.)
S. admonitor M.Proctor	approx. 68	4*x*	4*x*	4	1	3·172 ± 0·038
S. anglica Hedl.	51, 68	3*x*, 4*x*	4*x*	16	10	2·960 ± 0·038
S. aria (L.) Crantz	34	2*x*	2*x*	82	25	1·490 ± 0·017
S. cf. *aria*	–	–	3*x*	14	7	2·270 ± 0·028
S. aucuparia L.	34	2*x*	2*x*	13	10	1·522 ± 0·011
S. bristoliensis Wilmott	51	3*x*	3*x*	1	1	2·318
S. cambrensis M.Proctor	68	4*x*	5*x*	13	3	3·737 ± 0·050
S. cheddarensis L.Houston & Ashley Robertson	–	3*x*§	3*x*	5	2	2·264 ± 0·018
S. cf. *cheddarensis*	–	–	3*x*	15	4	2·265 ± 0·022
S. cuneifolia T.C.G.Rich	–	–	4*x*	1	1	2·963
S. devoniensis E.F.Warb.	68	4*x*	4*x*	6	3	3·151 ± 0·033
S. eminens E.F.Warb.	68	4*x*	4*x*	32	8	3·029 ± 0·018
S. cf. *eminens*	–	–	4*x*	3	2	3·028 ± 0·016
S. eminentiformis T.C.G.Rich	68	4*x*	4*x*	10	3	3·009 ± 0·027
S. eminentoides L.Houston	–	4*x*§	4*x*	5	1	3·002 ± 0·030
S. cf. *eminentoides*	–	–	4*x*	5	1	3·017 ± 0·020
S. hybrida L.	68	4*x*	4*x*	2	1	2·946 ± 0·053
S. intermedia (Ehrh.) Pers.	68	4*x*	4*x*	4	3	3·018 ± 0·054
S. lancastriensis E.F.Warb.	68	4*x*	4*x*	10	4	3·000 ± 0·029
S. latifolia (Lam.) Pers.	34, 68	2*x*, 4*x*	4*x*	1	1	3·143
S. leighensis T.C.G.Rich	–	3*x*§	3*x*	2	2	2·296 ± 0·000
S. leptophylla E.F.Warb.	68	4*x*	4*x*	5	1	3·007 ± 0·049
S. leyana Wilmott	51	3*x*	3*x*	4	1	2·265 ± 0·013
S. margaretae M.Proctor	68	4*x*	4*x*	6	4	3·014 ± 0·036
S. minima (Ley) Hedl.	51	3*x*	3*x*	4	1	2·275 ± 0·015
S. mougeotii Soy.-Will. & Godr.	68	4*x*	4*x*	5	3	3·018 ± 0·046
S. 'Observatory Hill taxon'	–	–	3*x*	12	1	2·255 ± 0·018
S. parviloba T.C.G.Rich	–	–	4*x*	1	1	3·011
S. cf. *parviloba*	–	–	3*x*	2	1	2·248 ± 0·004
S. porrigentiformis E.F.Warb. (*s.s.*)	68	4*x*	4*x*	23	9	3·018 ± 0·030
S. porrigentiformis (agg.)	–	–	4*x*	5	7	3·011 ± 0·015
S. porrigentiformis 'Symonds Yat clone'	–	–	3*x*	11	3	2·269 ± 0·017
S. cf. *porrigentiformis*	–	–	5*x*	1	1	3·807
S. 'Portishead taxon'	–	–	4*x*	19	4	3·016 ± 0·029
S. rupicola (Syme) Hedl.	68	4*x*	4*x*	10	4	3·068 ± 0·024
S. rupicoloides L.Houston	–	–	4*x*	3	1	3·013 ± 0·013
S. saxicola T.C.G.Rich	–	–	3*x*	3	1	2·247 ± 0·004
S. stenophylla M.Proctor	–	–	5*x*	5	2	3·696 ± 0·027
S. subcuneata Wilmott	51, approx. 68	3*x*, 4*x*	3*x*	12	7	2·346 ± 0·028
S. torminalis (L.) Crantz	34	2*x*	2*x*	14	7	1·612 ± 0·011
S. vexans E.F.Warb.	68	4*x*	4*x*	5	3	3·024 ± 0·030
S. wilmottiana E.F.Warb.	51	3*x*	3*x*	1	1	2·278
S. cf. *wilmottiana*	–	–	3*x*	1	1	2·275

* Chromosome numbers extracted from Rich *et al.* (2010) and the Index to Plant Chromosome Numbers (IPCN electronic database; http://www.tropicos.org/Project/IPCN).

† Ploidy inferred by FCM.

‡ Fluorescence intensity ratio (target sample peak/internal standard peak) = relative DNA content.

§ Ploidy inferred from molecular data (Houston *et al.*, 2009; Rich *et al.*, 2010; Robertson *et al.*, 2010).

Furthermore, the similar genome sizes found in the diploid sexual species (*S. aria*, *S. aucuparia*, *S. torminalis*), which are in agreement with the records of Siljak-Yakovlev *et al.* (2010) from the Balkan region, should be regarded as the main reason why the relative DNA contents fitted almost perfectly to ploidy allocations.

Ploidy diversity in Sorbus

The diploid chromosome number in *Sorbus* is $2n = 34$, in agreement with the currently accepted hypothesis that chromosome number in subfamily Maloideae arose via aneuploidy from $x = 18$ (Evans and Campbell, 2002). Chromosome counts in several species of the genus revealed the existence of di-, tri- and tetraploid taxa and one 'pentaploid' seedling (Bailey *et al.*, 2008; Rich *et al.*, 2010). The incidence of polyploid taxa in the investigated *Sorbus* is comparable with that reported in other related genera such as the above mentioned *Crataegus* (Talent and Dickinson, 2005) and other Rosaceae (Dickson *et al.*, 1992; Dickinson *et al.*, 2007). The present data confirm that diploidy is restricted to the parental taxa and their homoploid hybrids. Although Siljak-Yakovlev *et al.* (2010) recorded a triploid *S. torminalis* from Serbia, we have not found any polyploid specimen for the species in our sampling. In fact, and given the stability in ploidy of the British *S. torminalis* samples investigated, a hybrid origin for

TABLE 3. *Karyological information in known* Sorbus *hybrids*

Taxon	2*n**	Ploidy	Ploidy based on FCM	Ratio observed (mean ± s.d.)	Ratio expected	Difference (obs. – exp.)
S. × *thuringiaca* (*S. aucuparia* × *S. aria*)	34	2*x*	2*x*	1·505	1·506	–0·001
S. × *tomentella* (*S. aria* × *S. torminalis*)	34	2*x*	2*x*	1·552 ± 0·007	1·551	–0·001
S. × *avonensis* (*S. aria* × *S. porrigentiformis*)	–	3*x*†	3*x*	2·252 ± 0·058	2·250	0·004
S. × *liljeforsii* (*S. aucuparia* × *S. intermedia*)	51	3*x*	3*x*	2·349	2·270	0·079
S. × *robertsonii* (*S. aria* × *S. eminens*)	–	3*x*†	3*x*	2·271	2·260	0·011
S. × *houstoniae* (*S. aria* × *S. bristolensis*)	–	4*x*†	4*x*	3·072	3·063	0·009
S. × *motleyi* (*S. aucuparia* × *S. leyana*)	–	–	4*x*	3·039 ± 0·013	3·026	0·013

* Chromosome numbers extracted from Rich *et al.* (2010).
† Ploidy levels inferred from molecular data (Houston *et al.*, 2009; Robertson *et al.*, 2010).
Note: the ratios expected were calculated on the basis of the parental genomes. The calculations in diploid and triploid hybrids were made by summing the relative ratio of reduced genomes in both parents. In the tetraploids, the calculations resulted from the sum of an unreduced triploid and reduced diploid parents.

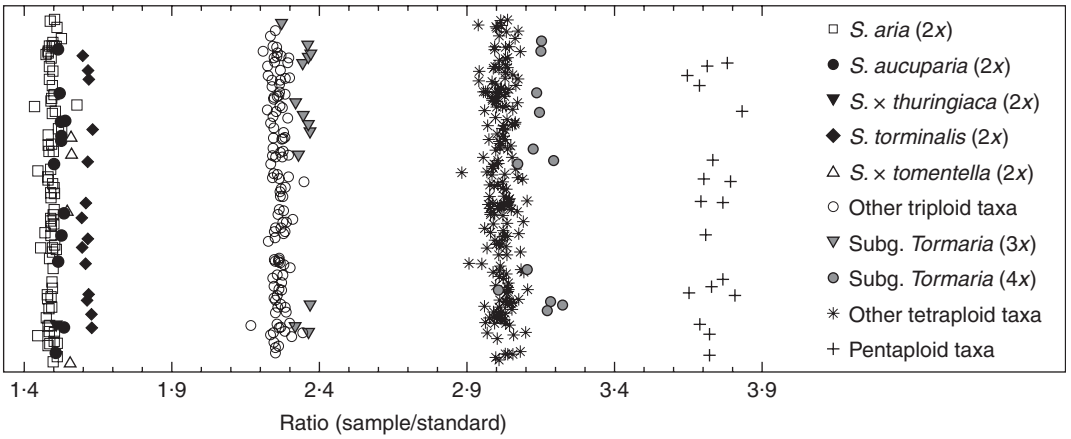

FIG. 4. Scatterplot of the flow cytometric data obtained illustrating the differences among ploidy levels in *Sorbus*.

this isolated triploid should be considered. Similar taxonomic issues have arisen when considering polyploid specimens of *S. aria*. Some authors still include the apomictic polyploids within *S. aria sensu stricto* (Aldasoro *et al.*, 2004), but with our species concept, *S. aria* is restricted to sexual diploid individuals.

Tetraploids and (to a lesser extent) triploids are the prevalent cytotypes of the apomictic taxa, accounting for most of the species richness in Britain. Additionally, although they are at low frequency, this extensive survey has confirmed the existence of pentaploid cytotypes in adult individuals at different sites (Supplementary Data Table S1), a cytotype previously only reported in a single seedling of the *S. porrigentiformis* group grown from Craig y Cilau (Wales). This illustrates the convenience of FCM for rapid ploidy screening from field conditions. As most apomictic *Sorbus* taxa use pollen for endosperm fertilization (pseudogamy), crosses involving different ploidies might prove problematic for endosperm balancing and sometimes result in seed abortion (Cosendai and Hörandl, 2010). This limitation could be the reason why pentaploids are poorly represented in *Sorbus*. However, given the high incidence of triploids in the data set and that some other pseudogamous Rosaceae (e.g. *Crataegus*; Talent and Dickinson, 2007) are not as sensitive to endosperm imbalance, other limiting factors cannot be discarded. Further investigation into the mechanism of apomixis in hybrid *Sorbus* would be rewarding in leading to improved understanding of how some cytotypes might occur, despite the disadvantages relating to endosperm formation.

Expected and unexpected results: new taxa or simple misidentifications?

Subgenus *Aria* is perhaps the most complex of the sections in Britain and it is not always possible to be certain of the identity from some of the immature or shaded plants that we sampled. This could explain why 14 specimens from seven sites collected as *S. aria* were 3*x* and not 2*x*, indicating that they are probably hybrids and only future molecular approaches will help to unravel their position within the *Aria* aggregate (Fig. 1). In addition, some potential new taxa were found: a triploid clone by the Observatory in the Avon Gorge, a triploid *S. porrigentiformis* relative from the Symonds Yat area and a stable tetraploid taxon from Portishead. This last named was suggested to be *S.* × *robertsonii* by Rich *et al.* (2010), but as their ploidy differs they must have different origins. Another interesting group of taxa is the *S. porrigentiformis* aggregate, in which we confirmed the existence of triploid and tetraploid cytotypes. The previously existing pentaploid count (2*n* = approx. 87) is more likely to

refer to *S. cambrensis*, which we have confirmed to be consistently pentaploid. As several species have already been segregated from the complex (e.g. *S.* × *cheddarensis*, *S. leighensis*, *S. saxicola*; Rich *et al.*, 2010), further molecular investigation would be useful in unravelling the aggregate and shedding light on the need (if any) for additional taxonomic rearrangements.

Another taxon in subgenus *Aria* in which conflicting cytotypes were found was *S. parviloba*. Its chromosome number is unknown but ploidy assessments revealed both triploid and tetraploid cytotypes. Given (1) the small population size of this endemic to Coldwell Rocks in Gloucestershire, provisionally categorized as 'critically endangered' *sensu* IUCN, and (2) our doubts in identifying two of the sampled trees, additional investigation will provide stronger evidence to confirm whether these are true established cytotypes or illustrate potential hybridization with neighbouring species. In addition, some trees in the Wye Valley previously regarded as backcrosses between *S.* × *tomentella* and *S. aria* (Price and Rich, 2007) were found to be triploid, in agreement with some limited nuclear microsatellite data (Bentham-Green, 2006).

Within subgenus *Soraria* there were also cases which are worthy of comment. *Sorbus* × *thuringiaca* nm. *pinnatifida* (=*S.* × *pinnatifida*) was originally believed to have arisen from a cross between *S. aucuparia* and *S. intermedia* (Fig. 1; Richards, 1975), but Nelson-Jones *et al.* (2002) rejected this assumption based on nuclear microsatellite results. The cytotype we inferred for the taxon evaluated (2*x*) correlates with its presumed origin (*S. aria* × *S. aucuparia*, cf. Rich *et al.*, 2010); however, further investigation is required to discard hypothetical multiple origins for this cultivated hybrid. *Sorbus anglica*, previously reported to occur at both triploid and tetraploid levels, was found to be consistently tetraploid despite collection of material from two of the three sites at which triploids had previously been reported (Bailey *et al.*, 2008). Robertson *et al.* (2010), based on molecular data, postulated that this species arose from a cross between *S. aucuparia* and a representative of the *S. porrigentiformis* aggregate (3*x*, 4*x*). Although our results are not conclusive, the sole cytotype found suggests that a triploid *S. porrigentiformis* cytotype would be involved in its origin, and that triploid chromosome counts reported might reflect seedlings having different ploidies to the parent from which the seeds were collected.

Hybrid taxa: how does FCM information help?

The hybrid origin for many *Sorbus* species has been frequently proposed (see Fig. 1 for illustration). However, few taxa have been taxonomically recognized as hybrids in Britain (Table 3; Rich *et al.*, 2010) due to the involvement of apomixis. One of the useful aspects of FCM is that it allows discrimination between taxa and their hybrids. In fact, the diploid hybrids studied yielded relative DNA contents similar to those of the non-hybrid assumed parents. These values were almost exactly intermediate (Supplementary Data Fig. S2B) and were in agreement with the ratios we calculated using the putative parents (Table 3). A similar situation was observed in the known triploid and tetraploid hybrids investigated. The high concordance between the values observed and predicted not only confirms their homoploid and polyploid hybrid origin, respectively, but also supports their parentage as postulated by morphological traits (Rich *et al.*, 2010) and molecular approaches (Nelson-Jones *et al.*, 2002; Chester *et al.*, 2007). However, the slight deviation observed in *S.* × *liljeforsii*, rather than being the result of genomic reorganization, could be influenced by the lack of complete agreement in the multiple origins suggested for the parental *S. intermedia* (Challice and Kovanda, 1978; Lemche, 1999). The ploidy survey we carried out also brought to light several specimens displaying unexpected ploidy, which were considered as potential interspecific crosses (Supplementary Data Table S1). In these cases, any inference about their origin based on the observed ploidy would be speculative, but provides new insight into the ability to hybridize in *Sorbus* and will be of great help in improving our understanding and interpretation of further research using molecular markers such as nuclear and plastid microsatellites.

Concluding remarks and future research

British *Sorbus* is revealed as an interesting example of a polyploid complex enhanced by recurrent hybridization episodes. A noteworthy coexistence of cytotypes (2*x*, 3*x*, 4*x* and 5*x*) has been found, probably facilitated by the overlapping distribution of many of the species studied, and resulting from the potential hybrid origin of many apomictic polyploids. As in former investigations, FCM has been found to be highly effective in estimating the relative DNA content of the species under study to infer ploidy. The data presented here provide a solid baseline for forthcoming molecular research aimed at gaining a better understanding of the genetic pathways controlling the formation and establishment of polyploids.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Fig. S1. Linear regression of the relative DNA ratios and DNA ploidy. Fig. S2. Distribution of the relative DNA contents (DNA ratios) in *Sorbus* grouped under different taxonomic criteria. Table S1: detailed information about the taxa studied including localities, collectors and flow cytometric results.

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