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The origin of intermediate species of the genus *Sorbus*

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Abstract The genus *Sorbus* in Europe contains five diploid species, *Sorbus aria*, *Sorbus aucuparia*, *Sorbus torminalis*, *Sorbus chamaemespilus* and *Sorbus domestica*, classified into five different subgenera. The subgenus *Aria* (or the *S. aria* aggregate) contains apomictic triploid and tetraploid species. Within the genus there are, in addition, a number of species, morphologically intermediate between plants of the four main subgenera, which are considered to reproduce partly, or exclusively, by apomixis. These are believed to have originated by hybridisation between species in the *S. aria* aggregate and a species from another subgenus (either *S. aucuparia*, *S. torminalis* or *S. chamaemespilus*). We have used restriction fragment length polymorphism (RFLP) analysis on a total of 178 *Sorbus* accessions to test this model. The genome relationships of the different groups have been assessed, and the hybrid nature of the major intermediate groups is unequivocally demonstrated. Polyploid species in the *S. aria* aggregate show genetic variation, indicating the possibility of multiple origins and/or facultative apomictic breeding behaviour. A major finding, confirmed by microsatellite analysis, is that the 'intermediate' species *S. intermedia* is shown to have genomes from *S aria*, *S aucuparia* and *S torminalis*. Polymorphic mitochondrial DNA markers were used to determine the direction of the crosses that gave rise to new 'hybrid species'; in the majority of cases the pollen was provided by the parent from the *S aria* aggregate.

Keywords Molecular markers · *Sorbus* · Polyploids · Origin of intermediates · Apomixis

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Introduction

Molecular approaches have resulted in many insights into the role of hybridisation and the recurrent origins of sexually reproducing polyploid groups of plants (Leitch and Bennett 1997). Nevertheless, much remains to be discovered about evolution in agamic complexes, in which some or all the members of polyploid groups reproduce by apomixis. This is where the seeds are produced without fertilisation and by processes that bypass the normal meiotic cycle (Hayward 1999). There has been renewed interest recently in apomictic plants, with some progress being made in unravelling the taxonomic (Campbell 1999), breeding behaviour (Gornall 1999) and evolutionary relationships within the 33 plant families that exhibit apomixis (Carmen 1997). Additionally, studies of the underlying genetic mechanism of apomixis have been undertaken, to explore the possibility of transferring apomixis genes into crop plants (Hayward 1999).

The genus *Sorbus* is a well-known example of an agamic complex. In Europe there are five diploid species, *Sorbus aria*, *Sorbus aucuparia*, *Sorbus torminalis*, *Sorbus chamaemespilus* and *Sorbus domestica*, classified into five different subgenera. The subgenus *Aria* includes not only the diploid sexual species (*S. aria sensu stricto* – hereafter *s.s.*), but also a number of triploid and tetraploid species. The whole group is known as the *S. aria* aggregate, and is often referred to as *S. aria sensu lato* (hereafter *s.l.*). Triploids have also been found in *S. chamaemespilus* (Liljefors 1955). As well as the species within these main groups, there are many species morphologically intermediate between the subgenera. These intermediate species are believed to have originated by hybridisation between species in the *S. aria* aggregate and either *S. aucuparia*, *S. torminalis* or *S. chamaemespilus* (Wilmott 1934; Liljefors 1955; Warburg and Kárpáti 1968; Richards 1975; Aas et al. 1994; Stace 1997). Most of these intermediates are triploid or tetraploid, each with a very restricted distribution (Hedlund 1948; Warburg and Kárpáti 1968). They have been proposed to reproduce partly, or exclusively, by apomixis

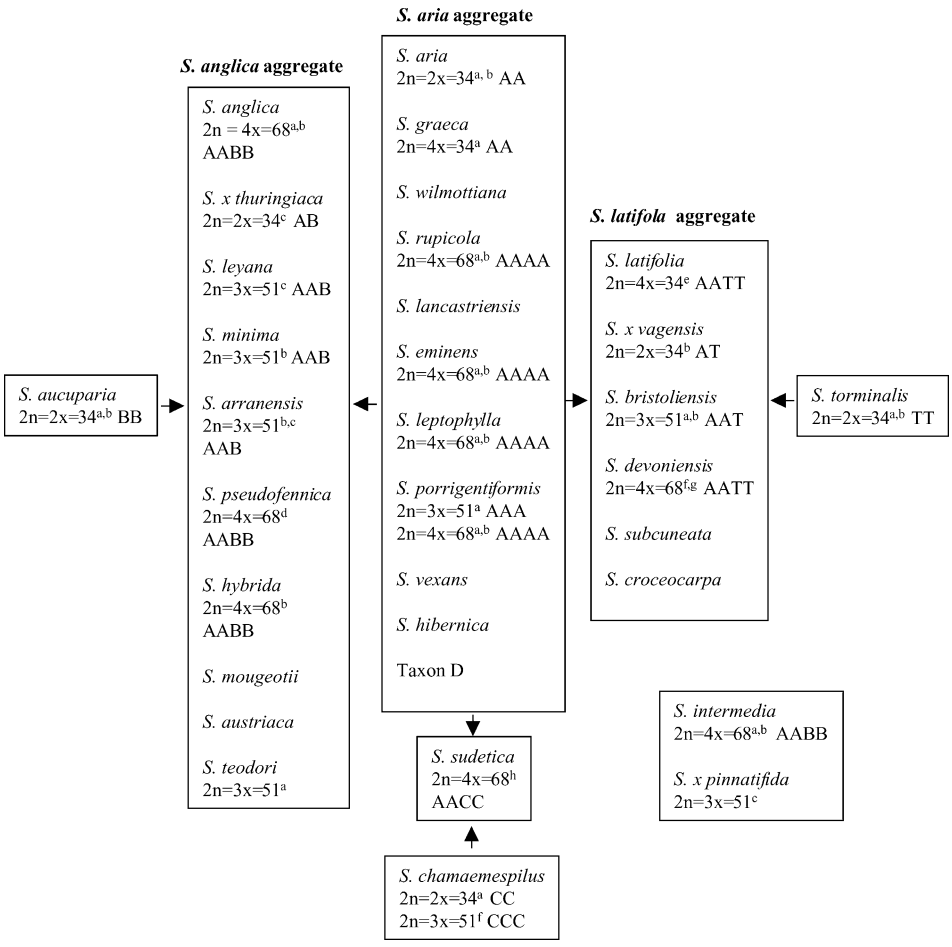

Fig. 1 Diploid and polyploid species of the genus *Sorbus* and the proposed relationships to intermediate species. The proposed genome composition (Liljefors 1955; Richards 1975) is indicated with *capital letters*: *A* = *aria* genome; *B* = *aucuparia* genome; *C* = *chamaemespilus* genome; *T* = *torminalis* genome. The chromosome numbers given are those that were available at the start of our investigations in 1996, most of which are in Floras or Compendia. Refer to Table 2 for new chromosome numbers that have been obtained since that date. References: [a] Warburg and Karpati (1968); [b] Clapham et al. (1987); [c] Richards (1975); [d] Hull and Smart (1984); [e] Pouques 1951; [f] McAllister (1986); [g] Sell (1989); [h] Liljefors (1955)

(Liljefors 1955; Richards 1975; Sell 1989; Proctor and Groenhof 1992). Figure 1 represents the species of these four subgenera and the intermediate species, together with the published chromosome numbers that were available when we started this work (but see below, and Table 2). Specific capital letters are commonly used to indicate the genomic composition and the ploidy level; for example the genome of the diploid species *S. aucuparia* is denoted BB. In those cases where the genome composition has been proposed by previous investigators (Liljefors 1955; Richards 1975), this is included in the figure.

Intermediate species can be divided into two large and two small groups. One large group, the *Sorbus latifolia* aggregate, consists of species intermediate in appearance between the proposed parent species *S. aria s.l.* and *S. torminalis* (Richards 1975; Proctor et al. 1989; Sell 1989; Aas et al. 1994). The other large group, the *Sorbus anglica* aggregate, contains species believed to originate from hybridisation between *S. aucuparia* and *S. aria s.l.* (Proctor et al. 1989). One of the small groups contains species intermediate between *S. chamaemespilus* and the *S. aria* aggregate. The last group of intermediate species contains the tetraploid species *Sorbus intermedia*. In this context, the hybrid *Sorbus* × *pinnatifida* must also be considered, as it is thought to be the cross between *S. intermedia* × *S. aucuparia* (Richards 1975).

Current theories about the origin of the different polyploid *Sorbus* species are mainly derived from taxonomic studies, using characters such as leaf shape, indumentum and venation, together with fruit shape and colour (Hedlund 1901, 1948; Wilmott 1934; Liljefors 1955; Richards 1975; Challice and Kovanda 1978a, b; Hull and Smart 1984; Sell 1989). This morphological evidence has been supplemented in some cases by chromosome counts, and by studies of the pairing of meiotic chromosomes in species and hybrids (Liljefors 1955), to interpret the genome composition (Fig. 1). Additionally, flavonoid glycosides (Challice and Kovanda 1978a, b) and peroxidase isozymes (Proctor and Groenhof 1992) have been used in the study of the complex. Evidence from the different approaches offers support for the theory of the hybrid origin for the majority of the intermediate species. However, for *S. intermedia* there is conflicting evidence. Originally it was believed to belong to the *S. anglica* group because of the shape of the leaves. However, studies of a range of morphological characters, as well as evidence from isozymes and flavonoid glycosides, indicate that *S. torminalis* may be one of the parents (Liljefors 1955; Challice and Kovanda 1978a, b; Proctor and Groenhof 1992).

Molecular methods are able to provide a more direct and critical means of establishing evolutionary relationships than traditional morphological approaches. Different

molecular techniques are useful for different taxonomic levels and problems (Hollingsworth et al. 1999). For example, sequencing of ribosomal DNA has been used in the study of distantly related plants (Chase et al. 1993), restriction fragment length polymorphisms (RFLPs) have proved invaluable in the study of related species, and microsatellites or slipped-strand repeats (SSRs) have proved useful in the study of variation within species (Ciofi et al. 1998).

In this study, DNA from British and selected European species of *Sorbus* has been subjected to RFLP analysis in order to access the origin of the intermediate species, including *S. intermedia*. Our aim was to test the hypotheses concerning the role of hybridisation and polyploidy in the evolution of the *Sorbus* group. We chose RFLP analysis because this approach targets nuclear DNA, and the RFLP fragments identified, usually one or a few polymorphic bands only, are inherited in a codominant fashion. Consequently, easily interpretable results are obtained that can be used to detect plants of hybrid origin by their additive profile. Thus, a new species arising from hybridisation (or indeed an individual plant of hybrid origin) can be expected to have bands characteristic of both parents (Soltis and Soltis 1989, 1991). In our studies of *S. intermedia*, further investigations were carried out using microsatellite analysis.

Having established the parent species for the intermediate species, our aim was to determine which species was the seed parent in each case. Whereas crosses are perhaps equally likely in both directions in outcrossing sexually reproducing species, this may not be the case in species complexes, if polyploids have a high level of apomictic reproduction. Accordingly, chloroplast (cpDNA) and mitochondrial DNA (mtDNA) patterns were examined in hybrids and their presumed parents. Although there was no variation seen in cpDNA, the mtDNA markers provided evidence to establish the direction of the crosses.

Materials and methods

Plant material

For the analysis of the relationship between the different British and selected European species of *Sorbus*, it was essential to have a wide range of material representing all of the described species. It was impractical to collect all the plants from the wild, so a large proportion of the material was obtained from botanical gardens, where there are collections of well authenticated British *Sorbus*, assembled and identified by experts (including H.A.McAllister, M.C.F.Proctor, P.D.Sell and E.F.Warburg).

The majority of the material was collected in spring from labelled trees in the following botanic gardens: Cambridge Botanic Garden; Wakehurst Place Garden; University Botanic Garden, Bristol; Royal Botanic Gardens, Kew; and Ness Gardens, University of Liverpool. Additionally, some material was collected from wild trees in the area around Bristol in Leigh Woods, and from the Avon Gorge. These plants were identified by Mrs. L. Houston, a local *Sorbus* specialist. The locations of individual trees were mapped and herbarium specimens were collected from fruiting trees in autumn. A total of 178 accessions (Table 1) was collected, representing all the species of the four subgenera and the 'intermediate species' shown in Fig. 1, except for *Sorbus* × *vagensis*, which was not available. Where possible replicates were used, and at least one specimen for each species was obtained from botanic gardens, where they had been identified and catalogued. Young leaves were harvested, brought to the laboratory, and quickly frozen in liquid nitrogen. Samples were stored at –70 °C prior to DNA extraction.

Pot-grown specimens that were available were used for chromosome counts on root tips, pretreated with α-bromonaphthalene on ice for 24 h, or hexachlorocyclohexane at room temperature for 3.5 h, to increase the mitotic index. The roots were fixed in absolute ethanol:glacial acetic acid (3:1, v/v) overnight, followed by hydrolysis in 1 M HCl at 65 °C for 8 min, before staining with Feulgen's stain. Chromosomes were counted under a phase-constrast microscope.

DNA extraction and RFLP analysis

All basic molecular biological methods were as described by Sambrook et al. (1989). To extract genomic DNA from the *Sorbus* samples, approximately 0.5–1.0 g of frozen leaf material was ground to a fine powder by vortexing in Falcon tubes in liquid nitrogen with two Biological Grinding Spheres (Boehringer Mannheim). DNA was extracted by the CTAB method of Gawel and Jarret (1991), up to the point of initial resuspension in 10 mM Tris–HCl, 0.5 mM EDTA, pH 8.0 (TE) buffer, and with the inclusion of an extra chloroform:isoamyl alcohol extraction. After resuspension in TE, the DNA was purified on caesium chloride gradients with a yield of approximately 25–100 μg of DNA per g of fresh leaf material.

For RFLP analysis, approximately 5 μg of DNA was digested to completeness with *Eco*RI (Boehringer Mannheim) and electrophoresed on a 0.8% agarose gel. Fragments were transferred to a Fluka type B nylon membrane (BioChemica) by Southern-blotting overnight, followed by washing in 2 × SSC. Membranes were wrapped in Saran Wrap and stored at 4 °C until use.

Prehybridisation of the membranes was carried out at 65 °C overnight in a solution of 5 × high salt buffer (3.0 M NaCl, 0.1 M Pipes, 20 mM EDTA, pH 8.0), Denhardts III (0.1% BSA, 1% Ficoll 400, 1% w/v polyvinyl pyrrolidone), sheared salmon sperm DNA (5 mg/ml) and water in the proportion 1:1:2:6. The [^{32}P]-labelled probe (see next section) was prepared from approximately 25 ng of the DNA fragment, using either the 'Rediprime' kit (Amersham Life Science) or the 'Ready to Go' kit (Pharmacia) in the presence of [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham Pharmacia Biotech). The probe was denatured with 0.3 M NaOH, and then added to the prehybridisation buffer. After hybridisation of the membranes overnight at 65 °C, the hybridisation buffer was discarded, and the membranes were washed with 6 × SSC, 0.1% SDS for 10 min at 65 °C, followed by three washes at room temperature with 0.1% SDS, 0.1 × SSPE. The blots were autoradiographed at –70 °C using Biomax MR film with an MS intensifying screen for 1–30 days, depending on the signal intensity.

Probes used for RFLP analysis

The probes used to hybridise to the Southern blots fell into three classes: (1) homologous *Sorbus* nuclear DNA probes; (2) nuclear DNA probes from heterologous plant species; and (3) probes for chloroplast and mitochondrial DNA (cpDNA and mtDNA):

(1) The *Sorbus* nuclear DNA probes, used in the majority of the blots, were obtained from a genomic library of DNA from *S. aria s.s.*, to ensure the probes would hybridise to the DNA from the majority of the plants examined, namely all the species in the *S. aria* aggregate, as well as intermediate plants. The library was constructed from 25 μg of DNA from *S. aria* that was digested to completion with *Pst*I, followed by size-fractionation on a sucrose gradient. Fractions containing fragments of 1.8–3.0 kb were ligated into *Pst*I-digested pBluescript-SK and transformed into *Escherichia coli* DH5α. Plasmids from a total of 100 white colonies picked at random, were isolated, digested with *Pst*I to remove the

Table 1 The *Sorbus* material used for DNA extractions. *Sorbus* refers to the species name assigned to the plant at the time of collection. The location column refers to where the samples were collected: Cam = Cambridge University Botanic Garden, Bris = University Botanic Garden Bristol; Ness = Ness Gardens, University of Liverpool; Wake = Wakehurst Place; Kew = Royal Botanic Garden Kew; Nat = collected in the wild. Most of these latter plants were collected around Leigh Woods and Avon Gorge. The exceptions are *S. intermedia*, which was collected in Wiltshire, and "No Parking", which was collected in the East Lyn Valley, Devon

Sorbus	PCO symbol[a]	Location							
		Cam	Bris	Ness	Wake	Kew	Nat	Total	
aucuparia	B	5	1	1	1	0	1	9	*anglica* aggregate
anglica	a	4	1	2	1	0	2	10	
x *thuringiaca*	b	1	0	0	0	0	1	2	
leyana	c	1	0	1	0	0	0	2	
minima	d	3	0	1	1	1	0	6	
arranensis	e	1	0	2	0	0	0	3	
pseudofennica	f	1	0	2	0	0	0	3	
hybrida	g	1	0	2	0	0	0	3	
mougeotii	h	0	0	1	0	0	0	1	
austriaca	i	0	0	1	0	0	0	1	
teodori	j	0	0	1	0	0	0	1	
aria	A	2	1	9	0	1	14	27	*aria* aggregate
graeca	r	1	0	2	0	0	0	3	
wilmottiana	k	1	0	1	0	0	3	5	
rupicola	l	1	0	5	1	1	0	8	
lancastriensis	m	1	0	7	1	0	0	9	
eminens	n	1	1	0	1	0	4	7	
leptophylla	o	1	0	3	1	0	0	5	
porrigentiformis	p	1	0	8	1	0	4	14	
vexans	q	1	0	1	1	0	0	3	
hibernica	s	1	0	1	0	0	0	2	
Taxon D	t	0	0	1	1	0	0	2	
latifolia	u	1	1	1	0	1	0	4	*latifolia* aggregate
bristoliensis	w	1	2	0	1	1	6	11	
devoniensis	x	3	0	5	1	0	0	9	
subcuneata	y	0	0	1	1	0	0	2	
croceocarpa	z	2	0	0	0	0	0	2	
torminalis	T	5	0	2	0	0	3	10	
sudetica	Su	0	0	1	0	0	0	1	
chamaemespilus	Ch	0	0	1	0	0	0	1	
intermedia	I	1	0	2	0	1	2	6	
x *pinnatifida*	xP	1	0	1	0	0	0	2	
E2	E2	1	0	0	0	0	0	1	
'No Parking'	N	0	0	1	0	0	1	2	
decipiens	D	1	0	0	0	0	0	1	
Total		44	7	67	13	6	41	178	

[a] PCO symbol = symbol assigned to the species in the principal coordinate analyses in Figs. 3 and 4.

insert, and then blotted onto nylon membranes as above. The filters were probed with [^{32}P]-labelled genomic DNA (prepared as above) from *S. aria* to identify those clones that produced weak signals. These represented low-copy DNA fragments, which would make good RFLP probes. A total of 11 such clones were identified.

(2) Heterologous nuclear DNA probes were of two types. The first was a region encoding part of the ribosomal DNA operon from *Brassica oleracea* (BOS8; Bennett and Smith 1991). Because the coding region for rRNA has been highly conserved throughout the angiosperms (Thompson et al. 1986), it was likely to hybridise to *Sorbus* DNA. The other group was a series of anonymous genomic DNA fragments from *Malus* spp., a gift from Dr. Graham King, Horticultural Research International, Wellesbourne, UK. *Malus* belongs to the same subfamily as *Sorbus*, and *Malus* probes could therefore be expected to hybridise with *Sorbus* DNA. Several genomic probes from *Malus* were tested, but only two (MS604 and MS134) produced informative results.

(3) In angiosperms, with few exceptions, inheritance of organelle DNA is through the maternal parent (Doebley 1992). Thus, using DNA from these organelles as probes can reveal the direction of the original hybridisation by identifying the seed-parent. Ten chloroplast probes representing the majority of the tomato (*Lycopersicon esculentum*) chloroplast genome (Phillips 1985) were obtained as a gift from Prof. J.C. Gray (Department of Plant Sciences, University of Cambridge). Two mitochondrial DNA fragments encoding cytochrome oxidase subunit I (*COXI*) and the α-subunit of the ATP synthase (*atpA*) from maize (*Zea mays*) were obtained from Prof. C.J. Leaver (Department of Plant Sciences, University of Oxford).

Microsatellite analysis

Primers for microsatellites developed for *Malus* were obtained from Dr. Graham King, HRI, Wellesbourne. Two that gave reproducible results with *Sorbus* DNA were MS6g (forward: 5′CGGAGGGTGCTGCCGAA3′ and reverse: 5′GCCCAGCCCATATCTGCT3′) and MS14 (forward: 5′CGCTCACCATCGTAGACGT3′ and reverse: 5′ATGCAATGGCTAAGCATA3′). The primers were labelled with [γ-^{33}P]ATP (≥2,500 Ci/mmol; Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Boehringer Mannheim). PCR reac-

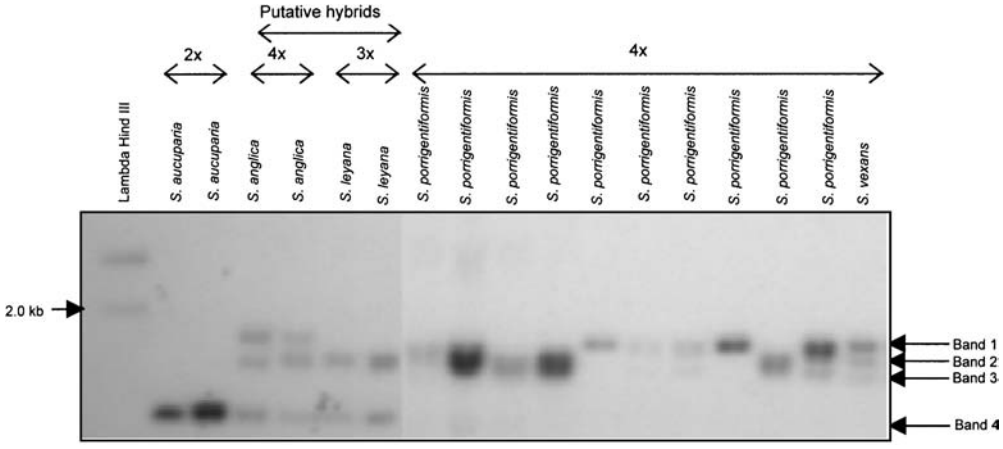

Fig. 2 Southern blot of DNA from S. *aucuparia*, species from the *S. aria* aggregate and their putative hybrids, probed with an anonymous genomic probe from *Sorbus*. The ploidy level is indicated at top of the figure. On the left-hand side the 2.0-kb lambda-*Hin*dIII marker is *arrowed*. On the right hand side, *bands 1–3* are found in the samples from the *S. aria* aggregate, while *band 4* is characteristic of *S. aucuparia*. The hybrids contain bands from both putative parents

tions were carried out in a total volume of 20 µl with 20 ng of template DNA, 1.5 mM of $MgCl_2$, 20 nM of each primer, 0.5 units of *Taq* polymerase (Bioline), 1 × buffer (NH_4^+), 0.2 mM of dNTPs. Reactions were carried out for 35 cycles with annealing temperatures of 54 °C for MS6g and 53 °C for MS14. Products were analysed on a 6% polyacrylamide sequencing gel in the presence of urea, and autoradiographed against Biomax MR film for 7 days.

Data analysis

Individual bands from the RFLP analysis were scored as present or absent for each DNA sample, and polymorphic bands were compiled into a binary matrix. Comparisons between DNA samples on different gels were made possible by the use of duplicate samples on two or more gels. Only bands that could be reliably scored on all gels were included. Markers from mitochondrial probes were analysed separately from those obtained with nuclear DNA probes.

The binary matrix from the nuclear probes was imported into the program GenStat5, and into the Multi-Variate Statistics Package (MVSP; Kovach 1993). The distance for all individuals in the matrix was calculated using Jaccard's Coefficient: $Jc_{ij} = a/(a+b+c)$, where Jc_{ij} is the similarity in variables i and j, a = number of bands shared, b and c = number of fragments only in individuals b and c respectively. A similarity matrix was then derived using the standard algorithm, in brief: the two closest individuals are identified and 'fused' to form a similarity node, and the matrix reduced by one. The reduced matrix is then reanalysed and the process repeated until all individuals have been fused. This similarity matrix was used for a Principal Coordinate Analysis in MVSP.

Results

Specimen collection and chromosome counts

To ensure that our results were as meaningful as possible, the collection of *Sorbus* specimens was designed to sample as widely across the species present in Britain, ensuring that for each we had replicates and at least one sample from a botanic garden (see Table 1). For those species for which pot-grown material was available, we determined the chromosome number in root tips. Our results are shown in Table 2, together with the previously published values (shown in Fig. 1), and those that were made public during our work. In general our findings confirmed those of other workers, except for *Sorbus vexans* and *S. latifolia*, which we found reproducibly to have 51 and 68 chromosomes, respectively. Clearly, the chromosome composition in *Sorbus* is complex, with intraspecific variability observed for a number of species. This may reflect the variability in the wild, or there may be errors in the counts. The situation in *Sorbus* is representative of a general problem concerning the reliability of published chromosome numbers. For example, for *myosotis* species many chromosome numbers were found to be erroneous for several different reasons (Merxmüller 1970). Thus difficulties may arise from

Table 2 Somatic chromosome numbers of *Sorbus* species. Determinations were made in pot-grown specimens of as many species as available. The published chromosome numbers available at the start of the work (1996), as shown on Fig. 1 are included, together with those made available by other workers since that date. References a–h are as in Fig. 1. [i]Stace 1997; [j]http://www.bsbi.org.uk; [k]H.A. McAllister (University of Liverpool), personal communication; [l]P.D. Sell (University of Cambridge), personal communication

Sorbus	Chromosome counts		
	Our work	Reported prior to our investigations	Numbers reported subsequently
aucuparia	34	34[a,b]	34[j]
anglica	68	68[a,b]	68[j]
leyana	51	51[c]	–
minima	51	51[b]	–
arranensis	51	51[b,c]	–
pseudofennica	–	68[d]	51[i]
aria	34	34[a,b]	–
wilmottiana	51	–	51[i]
rupicola	68	68[a,b]	34, 51[i] or 68[j]
lancastriensis	68	–	68[l,j]
eminens	–	–	34, 51[i,j]
leptophylla LP4	68	68[a,b]	51, 68[i,j]
porrigentiformis	–	51, 68[a] or 68[b]	34, 68[i,j]
vexans	51	–	68[i]
Taxon D	68	–	68[k]
latifolia	68	34[e]	51[i] or 68[j]
bristoliensis	51	51[a,b]	–
subcuneata	–	–	51, 68[l]
croceocarpa	68	–	–
intermedia P	68	68[a,b]	–

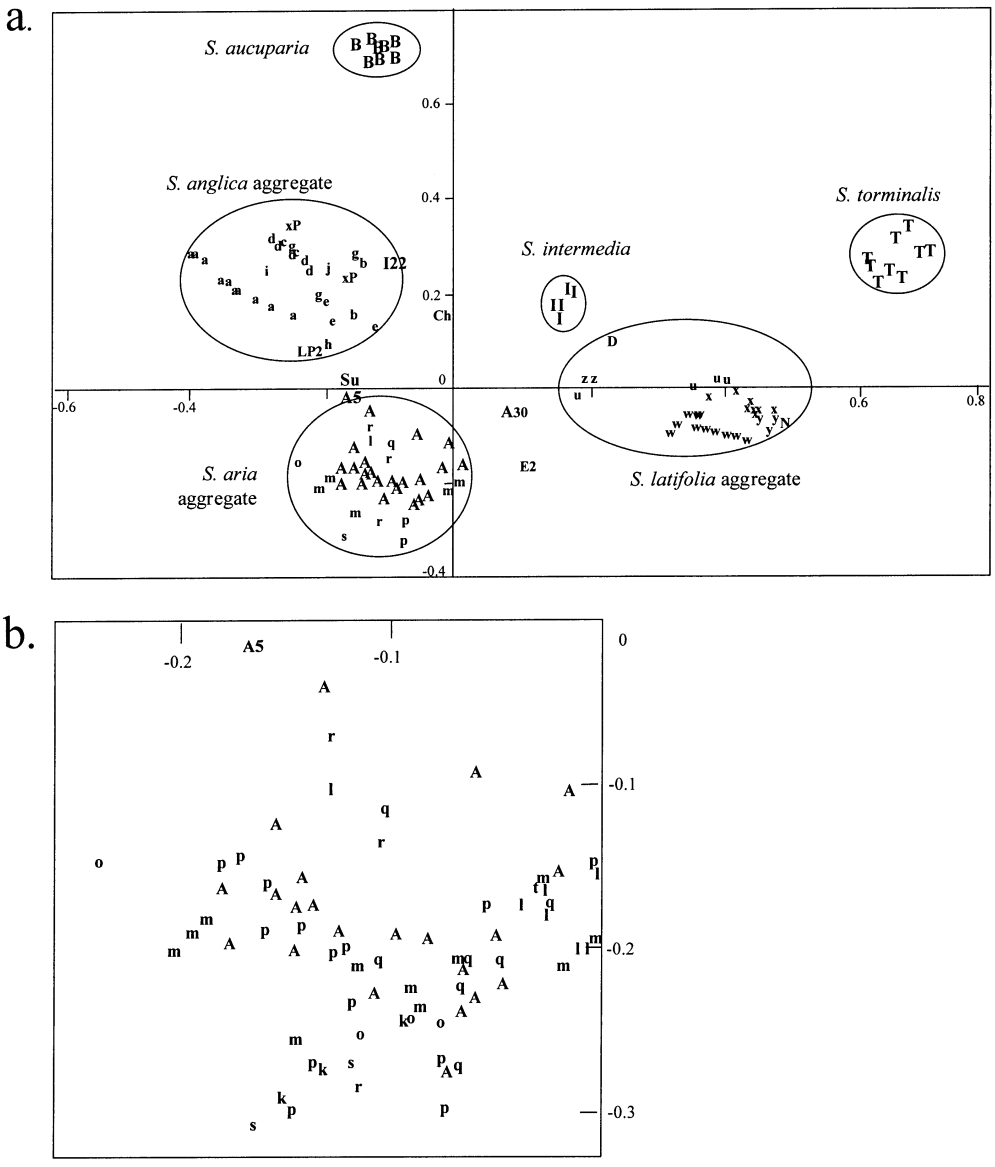

Fig. 3a, b Principal coordinate analysis (PCO) of all the polymorphic bands identified in the RFLP analysis, showing the first and second axes. These together constitute 23.4% of the observed variation. **a** The complete PCO analysis showing the distribution of all the species. The major groupings (Fig. 1) are *circled*. Samples atypical of their presumed group have been *labelled* individually (*aria* A5, A30, E2; *intermedia* I22; *leptophylla* LP2). For clarity only a proportion of the individual specimens are included in the *S. aria* aggregate. **b** An expansion of the region of the PCO containing the *S. aria* aggregate showing all the specimens. Key:

aucuparia	B	*aria*	A	*latifolia*	u	*sudetica*	Su
anglica	a	*wilmottiana*	k	*bristoliensis*	w	*chamaemespilus*	Ch
x *thuringiaca*	b	*rupicola*	l	*devoniensis*	x	*intermedia*	I
leyana	c	*lancastriensis*	m	*subcuneata*	y	*x pinnatifida*	xP
minima	d	*eminens*	n	*croceocarpa*	z	E2	E2
arranensis	e	*leptophylla*	o	*torminalis*	T	No Parking	N
pseudofennica	f	*porrigentiformis*	p			*decipiens*	D
hybrida	g	*vexans*	q				
mougeotii	h	*graeca*	r				
austriaca	i	*hibernica*	s				
teodori	j	Taxon D	t				

misidentification and/or mishandling of samples. There may be differences of opinion about the status of particular specimens, and only small numbers of cells were counted in one or a few (perhaps atypical) plants. Furthermore, in critical genera, where interspecific hybridisations occur, the use of progenies to infer the chromosome number of seed parents may be difficult, especially in seed collected from mixed populations of several spe-

cies in the wild or in gardens. In addition, in *Sorbus*, there is a long time lag between the seedling phase in which mitotic chromosome counts are made, and the sexually mature plant with flowers and fruits necessary for an accurate confirmation of identity.

Principal co-ordinate analysis of RFLPs from nuclear DNA probes

For the RFLP analysis, each *Sorbus* sample was analysed on at least two different Southern blots for each probe. This had the dual purpose of making it possible to compare band sizes from different autoradiographs, and provided a control for scoring the bands. The polyploid species of *Sorbus* are thought to reproduce apomictically. If this were the case and species were only formed once, then very little variation would be expected within the species, but species in the *aria* aggregate proved to be variable. An example of this is shown in Fig. 2, which is a Southern blot of DNA from polyploid species from the *aria* aggregate, together with two putative hybrids between *S. aria s.l.* and *S. aucuparia*. Band 4 is characteristic of *S. aucuparia*, whereas bands 1–3 are found in *S. porrigentiformis* and *S. vexans*. Clearly, more variation is evident within the latter two species than in the putative hybrids, which indicates the possibility of multiple origins from genetically different parents and/or that some sexual reproduction was occurring.

A total of 119 polymorphic bands obtained from the RFLP analyses was used for principal co-ordinate analysis (PCO; Kovach 1993), as described in the Materials and methods. The results for the first and second co-ordinates, accounting for a total of 23.4% of the variation, are shown in Fig. 3a. Each species is assigned a letter symbol, also indicated in Table 1. However, not all the samples of the *S. aria* aggregate could be displayed easily on this figure, and so an enlarged version of this region is shown in Fig. 3b. It can clearly be seen in Fig. 3a that *S. aucuparia* (denoted B), *S. torminalis* (denoted T) and the *S. aria* aggregate form distinct groups. The individual specimens of the different species within the *aria* aggregate are widely spread out (Fig. 3b), and show more variation than is seen in either of the diploid species *S. aucuparia* or *S. torminalis*. Further investigation is needed to discover how far this is due to the small sample size of these diploids (although see Fig. 4 below). The *S. anglica* aggregate is located between *S. aria s.l.* and *S. aucuparia*, supporting the hypothesis that plants in this group originate from hybridizations between them. Similarly, the position of the *S. latifolia* aggregate between the *S. aria* aggregate and *S torminalis* indicates that *S. latifolia s.l.* has the hybrid origin *S. aria s.l.*. × *S. torminalis*. In contrast, a specimen of the central European species *S. chamaemespilus* (Ch) is not included in any of the major groupings. The species *S. sudetica* (Su) is located between *S. chamaemespilus* and species in the *S. aria* aggregate, a finding that supports the hypothesis that it originated from hybridization between

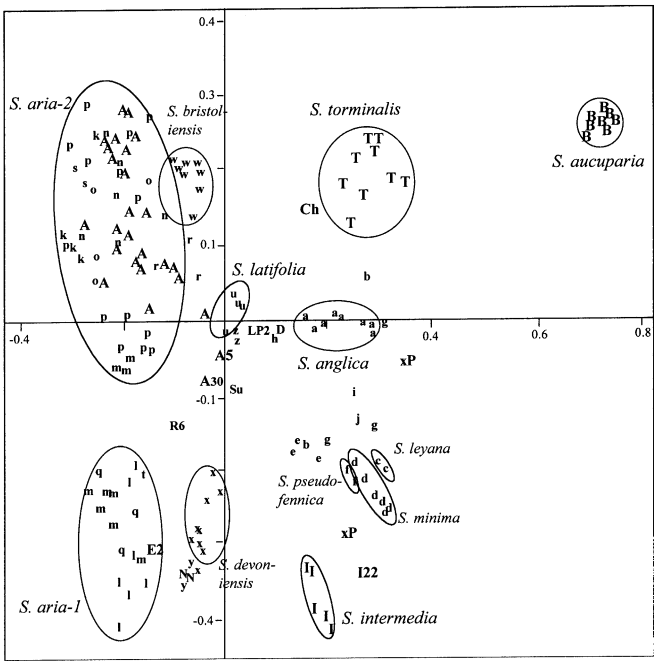

Fig. 4 PCO analysis showing the second and third axes, representing a total of 18% of the observed variation. Key as for Fig. 3, with the addition of R6 = *rupicola* sample. Where there are defined groupings of intermediate species, these have been *ringed and named*. The *S. aria* aggregate has been divided into two groups, with a few individual specimens outside, but these have not been named as they both contain samples from several species

these two groups (Hedlund 1901; Jankun and Kovanda 1987).

Figure 4 shows the 2nd and 3rd axes of the PCO (18% of variation). The three subgenera are again separated, but now the species in the *S. aria* aggregate can be seen to fall into two quite distinct clusters, and several more accessions do not group closely with either set. In the intermediate aggregates most of the specimens have been assigned the same name cluster together. This suggests that these species are well defined, of a single origin and/or obligately apomictic. However, the species *Sorbus devoniensis* (x) and *Sorbus subcuneata* (y), and the "No Parking Tree" (N) are not well separated, raising the possibility of some sexual reproduction within the group. In the *S. aria* aggregate the named species do not form clear clusters, as would be expected if the plants were exclusively reproducing apomictically, suggesting the possibilility that some sexual reproduction has occurred, and/or the species has multiple origins. The lower subgroup of the *aria* aggregate (*S. aria*-1) consists of the majority of the *Sorbus lancastriensis* (m), *S. vexans* (q) and *Sorbus rupicola* (l) specimens, whereas the upper group (*S. aria*-2) comprises most of the remaining. From the position of the intermediate species along the 3rd axis, the *aria* group from which they are descended can be inferred. Thus, one of the parental species of *Sorbus bristoliensis* (w) would have belonged to *S. aria* group 2, while the ancestor of *S. devoniensis* (x) and *S. subcuneata* (y) came from group 1.

Table 3 RFLP analysis with mitochondrial DNA probes. Three band patterns were observed with the maize *COXI* probe, and two with the *atpA* probe. For each species, all specimens gave identical band patterns except for those indicated with an asterisk, followed by numbers in brackets, which indicate the number of specimens with this pattern. The *COXI* probe separates the samples into three groups, corresponding to each of the three subgenera, *Aucuparia*, *Torminalis* and *Aria*. The *atpA* probe gives identical band patterns in all species, except for a subset in the *Aria* subgenus

	COXI Pattern 1	*COXI* Pattern 2	*COXI* Pattern 3
atpA Pattern 1	*aucuparia* *anglica* *S.* × *thuringiaca* *leyana* *minima* *arranensis* *pseudofennica* *hybrida* *mougeotii* *austriaca* *teodori* *intermedia* *S.* × *pinnatifida* **leptophylla* LP2 (1) **aria* A5 (1)	*rupicola* *lancestriensis* **porrigentiformis* (6) **latifolia s.s.* (3) *vexans* Taxon D E2 *sudetica* **leptophylla* (2) **aria* (6)	*torminalis* **latifolia* (1) *bristoliensis* *devoniensis* *subcuneata* *croceocarpa* *decipiens* **aria* 30-94 (1)
atpA Pattern 2		**aria* (19) **leptophylla* (2) **porrigentiformis* (8) *wilmottiana* *chamaemespilus* *eminens* *graeca* *hibernica*	

In Fig. 3a, five specimens of *S. intermedia* (I) form a separate group in the middle of the three subgenera (*S. aria, S. torminalis* and *S. aucuparia*), between the *S. anglica* and *S. latifolia* aggregates. Similarly, on Fig. 4, the same five *S. intermedia* specimens are quite clearly separated from the other intermediate species. It seems possible therefore, that the group arose by hybridisation between the three subgenera. In order to test this hypothesis, it was necessary to use a technique that could distinguish genetic variation at higher resolution, and so we used microsatellite analysis, using primer sets developed for *Malus*. Several primer sets were tested, but only two (MS6g and MS14) gave reproducible bands with *Sorbus* DNA, and then only with two of the subgenera, and their hybrids; no clear signals were obtained using DNA from species in the *S. aria* aggregate. Nevertheless, from this analysis we were able to demonstrate that *S. intermedia* shared two bands with both *S. torminalis* and *S. latifolia* (data not shown), providing supporting evidence that *S. torminalis* was one of the original parents of *S. intermedia*.

Mitochondrial and chloroplast probes

In order to determine the direction of the original hybridisations we attempted to identify the seed-parent using organelle DNA as probes, since this is maternally inherited. We tested several fragments that together covered most of the chloroplast genome from tomato. However, they produced identical RFLP patterns for all the *Sorbus* samples and were not studied further. In contrast, the maize mtDNA probes proved more successful, as might be expected since mtDNA is more variable than cpDNA (Palmer 1992). Three polymorphic bands were obtained with the *COX1* probe, which divided the material into three groups, *S. aucuparia*, *S. torminalis* and the *S. aria* aggregate (Table 3). It is worth noting that *S. intermedia* has the *S. aucuparia* type *COX1*, while *S. chamaemespilus* has the *S. aria* type *COX1*. Among the intermediate species it is noticeable that only *S. sudetica* (both putative parents have the same *COX1* type) and three specimens of *S. latifolia* have the *S. aria* COX1 band. All the remaining intermediates examined, as well as the presumed direct hybrid *Sorbus* × *thuringiaca*, have either the *S. aucuparia* or the *S. torminalis COX1* bands. The *atpA* probe was less variable, with the majority of specimens having the same RFLP pattern, with another pattern being found only some of the species in the *Aria* subgenus. Interestingly, however, for three of the species (*aria, leptophylla* and *porrigentiformis*) both patterns were found. This provides further evidence that these species had multiple origins.

Discussion

Our molecular studies have been designed to test a number of hypotheses that have emerged from more than a century of taxonomic and experimental observations. However, it is important to stress that our findings have to be interpreted against a background of incomplete information, in particular about chromosome numbers and the degree to which polyploid *Sorbus* species are obligate or facultatively apomictic (see below).

Nevertheless, the data we have obtained from RFLP analysis has proved invaluable in confirming the postulated groupings within the genus *Sorbus*; namely the four subgenera, and the hybrid origin of the *S. anglica* and the *S. latifolia* aggregates. In general, it has also con-

Table 4 Origin of European species of *Sorbus* determined from the PCO analysis (Figs. 3 and 4). The table lists the distribution of the intermediate species together with their proposed origins, as indicated in Flora Europaea [FE] (Warburg and Kárpáti 1968) and Stace (1997) [ST]

Species	Distribution	Proposed origin tested by PCO analysis
S. austriaca	Eastern Alps, Carpathians, Balkans	Related to *S. mougeotii* [FE] (see below)
S. chamaemespilus	Mountains of Central and Southern Europe	Distinct diploid species; as expected, found outside the main clusters in PCO
S. croceocarpa	Origin unknown	PCO analysis supports the proposal [ST] that the species arose from the cross *S. aria S. torminalis*
S. decipiens	Central Europe	Proposed origin [FE] *S. aria* group or related species × *S. torminalis* supported by PCO analysis
S. graeca	SE and EC Europe	Affinities with *S. aria* group [FE] confirmed by PCO analysis
S. mougeotii	Alps, Pyrenees	Position in PCO analysis supports proposed origin [FE] from *S. aucuparia* × *graeca*
S. × *pinnatifida*	Introduced in Britain or found with parents [ST]	PCO analysis does not support the proposed origin [ST] *S. aucuparia* × *S. intermedia*
S. sudetica	Czechoslovakia	Proposed origin [FE] *S. chamaemespilus* × *S. aria* (either species, group or relative). In PCO analysis samples positioned between *S. chamaemespilus* and *S. aria*
S. teodori	Sweden	1. *S. aucuparia* × *S. rupicola* or 2. *S. aucuparia* × *S. chamaemespilus* [FE]. Not resolved in PCO analysis

firmed the groupings in Fig. 1, including the fact that *S. chamaemespilus* (Ch) is a distinct diploid species, found outside the main clusters in the PCO analysis. Similarly, from their positions in the PCO, it is possible to draw firm conclusions about the origins of most of the mainland European intermediate species (Table 4). Nevertheless, there are a number of exceptions, which are discussed further below. Additionally, although specimens of the same species are generally close together in the PCO analyses of nuclear DNA markers, this is not always the case. The most notable example is *S. porrigentiformis* (p), specimens of which are as widely scattered as those of *S. aria s.s.* (A) and indeed are much more variable than the other diploid species *S. torminalis* (T) or *aucuparia* (B). Interestingly, however, all *S. porrigentiformis* specimens are found in the upper *S. aria* ggregate on Fig. 4. In contrast specimens of *S. lancastriensis* (m) are found in both clusters.

The position of *S. intermedia* (I) in the PCO graph in Fig. 3, between *S. aria s.l.*, *S aucuparia* and *S torminalis*, supports the theory that all three genomes are somehow combined in this species, making the name *S. intermedia* particularly appropriate. The microsatellite data provided support for *S. torminalis* being amongst the parents of *S. intermedia*, whilst unequivocal molecular evidence for the parentage came from the demonstration that *S. intermedia* has the *S. aucuparia* mitochondrial type (Table 3). These data support the proposal that *S. intermedia* originated from a cross involving an unreduced gamete from a triploid plant in the *S. anglica* aggregate and a reduced gamete from *S. torminalis* (McAllister 1986). Thus, *S. intermedia* would have the genomic constitution AABT.

S. intermedia has been regarded as facultatively sexual as it had high pollen fertility (20% germination percentage in 15% sucrose solution), and almost regular chromosome pairing (Liljefors 1953). Furthermore, it proved possible to produce the artificial hybrids *S. intermedia* × *S. aucuparia* and *S. intermedia* × *Sorbus hybrida* (Liljefors 1953, 1955). However, this does not prove that *S intermedia* can produce *S intermedia*-like plants sexually. Further work will be necessary to resolve these issues, as, following embryological studies, Jankun (1994) concluded that *S. intermedia* is an obligate apomict.

If *S. intermedia* is a three-way hybrid with the unbalanced genome AABT it is not obvious how the chromosomes pair at meiosis. One possibility is that the two 'A' genomes pair and that the genomes 'T' and 'B' form bivalents. If this is the case, *S. intermedia* × *S. aucuparia* (synonymous with *S.* × *pinnatifida*; Richards 1975) must have the genome composition AAB or ABT. Liljefors (1955) concluded, on the basis of a very irregular chromosome pairing dominated by univalents, that *S. intermedia* × *S. aucuparia* was most likely to have the genome combination 'ABT'. However, if the specimens of *S.* × *pinnatifida* (xP) have the genome combination 'ABT', they would have been expected to group with *S. intermedia* in the PCO (Fig. 3), whereas they grouped with the *S. anglica* aggregate. One explanation for this is that the two specimens of *S.* × *pinnatifida* included in the analysis were not correctly identified. Another possibility is that the sexual genomes from *S. intermedia* can be either AT or AB and the hybrid *S. intermedia* × *S. aucuparia* can have either the genome combination ABT or ABB. It is likely that Liljefors (1955) only studied one or very few specimens of *S. intermedia* × *S. aucuparia* and, even if ABB exists, he may only have detected ABT.

A third and possibly more straightforward explanation of the sexuality of *S. intermedia* is that the DNA

from *S. torminalis* and *S. aucuparia* has become integrated into the genome of *S. intermedia* and is no longer divided into distinct genomes. This could increase the level of regular chromosome pairing and the possible production of fertile gametes. If the genomes of the *S. torminalis* and *S. aucuparia* are indeed integrated in *S. intermedia*, it could explain the few bivalents formed in meiosis in the hybrid *S. intermedia* × *S. aucuparia* (i.e. *S.* × *pinnatifida*).

Another clear result from the RFLP analysis is that two specimens, *S. leptophylla* LP2 (LP2) and *S. intermedia* I22/2 (I22), have been mislabelled in the Botanic Garden collections. *S. leptophylla* LP2 is found in the *S. anglica* aggregate and not as expected within the *S. aria* group. Misidentification of the specimen has most likely occurred because it has entire leaves, a character typical of both *S. anglica* and species in the *S. aria* group. Several of the specimens in the *S. aria* aggregate (including *S. leptophylla*) are said to be variable (Proctor and Groenhof 1992). The specimen labelled *S. intermedia* I22/2 is grouped with the *S. anglica* aggregate rather than with the other *S. intermedia* plants. This suggests that the plant may have been wrongly identified as *S. intermedia*. Indeed, when it was collected, a note was made that the leaves were more pinnate (more *S. hybrida*-like) than expected for *S. intermedia*.

In contrast to the clear clustering of most of the specimens, there are a few that are ungrouped in the PCO analysis (Figs. 3 and 4). The specimens *S. aria* 30-94 (A30) and E2 are placed between the *S. aria* and the *S. latifolia* aggregates. Similarly, the specimen *S. aria* A5 is positioned between the *S. anglica* aggregate and the *S. aria* aggregate. An explanation for inter-group positions for these plants could be unbalanced genome compositions, where the majority of the genome comes from *S. aria s.l.* and only a single chromosome set originated from *S. torminalis* or *S. aucuparia*. Indeed, it has been suggested that *S. anglica* has an unbalanced genome because the leaves of *S. anglica* are less deeply lobed than those in any of the other British species in the aggregate (McAllister 1986). If an unbalanced genome composition does not generally result in a position between intermediate plants with a balanced genome and the parent that dominates the unbalanced genome, it seems unlikely to be the explanation for any of the inter-group positions. Another explanation for the inter-group positions could be that the different genomes have not remained distinct within the hybrid species and that some recombination has taken place, so that intermediate plants derived from other intermediate plants do not have a full genome of one of the original parents. This explanation would match the proposed integration of the parental genomes in *S. intermedia*.

An interesting observation from the study of mitochondrial DNA composition (Table 2) is that the majority of the intermediate specimens have the mitochondria type of either *S. aucuparia* or *S. torminalis*. This suggests that it is more likely for a hybrid to be formed with *S. aria s.l.* as the paternal parent than as the maternal parent, but it is clearly not a physiological requirement, because there are exceptions, such as *S. sudetica* and three of the four *S. latifolia* specimens. The reason that the polyploid hybrids predominantly have *S. aria s.l.* as the paternal parent may instead be related to whether members of the different subgenera have a different potential for producing diploid gametes in pollen and ovules.

Polyploid intermediate plants, with *S. aria s.l.* as the paternal parent, may either originate from pollen with an unreduced gamete from the diploid *S. aria s.s.*, or from sexually reduced pollen of a tetraploid species in the *S. aria* aggregate. Jankun and Kovanda (1988) claim to have found a tendency for apomixis in a diploid *S. aria s.s*. Thus, it is possible that the apomictic gene(s) may come from *S. aria s.s*. However, while the gene(s) may exist, it (they) may not be very common. Another possibility is that diploid pollen, originating from a tetraploid plant in the *S. aria* aggregate, is likely to carry the gene(s) for apomictic reproduction. Therefore, sexually reduced pollen from tetraploid members of the *S. aria* aggregate is most likely to be the common mode of origin of the polyploid intermediate species.

In conclusion, we have demonstrated the efficacy of the use of molecular markers to disentangle some of the evolutionary relationships between species in a complex genus such as *Sorbus*. For a more complete picture it will be necessary to have chromosome counts for population samples Europe-wide, and molecular, embryological and crossing experiments must be carried out to examine population variability in seed-parents and progeny (produced both in artificial crosses and by open pollination). Only then will it be possible to understand the breeding behaviour of different taxa, and allow a proper assessment of the patterns of apparent hybridisations and of recurrent polyploidy.

The origin of *S. intermedia* detected in our investigation demonstrates a mechanism by which apomictic genes in one species might facilitate the integration of genes from different non-apomictic species, and thereby increase the potential for long-term survival of hybrid derivatives. Our results suggest the possibility that genomic recombination and integration might occur in the evolution of new polyploids and that such integration might increase their potential for sexual reproduction. The question of genomic recombination is an important issue in considering the evolution in the genus over a longer time-frame, for recent studies of isozymes supports the hypothesis of an allopolyploid origin of *S. aucuparia* and the subfamily Maloideae (Raspé et al. 1998).

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