

## Parentage of an unknown member of the *Sorbus latifolia* (Lam.) Pers. group (Rosaceae)

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### ABSTRACT

Comparative morphology, AFLP fingerprinting and *trnL-F* region sequencing were used to investigate the origin of an unknown member of the *Sorbus latifolia* group with weakly lobed leaves and large fruits, discovered at Naas near the River Severn, Gloucestershire. Samples of the unknown hybrid, the three putative parents *S. aria*, *S. rupicola* and *S. torminalis*, and from *S. × vagensis*, the primary diploid hybrid between *S. aria* and *S. torminalis*, were analysed. Morphological analysis of leaf characters showed that the unknown hybrid fell between *S. aria* and *S. torminalis*. Genetic fingerprints were obtained using AFLP, and two primer combinations gave 109 bands, 84 of which were polymorphic. Both hybrids showed additivity between *S. torminalis* and *S. aria/S. rupicola*. *Sorbus torminalis* was one parent of both hybrids. The presence of twelve unique bands in *S. rupicola* (compared to only three bands in *S. aria*) indicate that *S. aria* is probably the other parent in both hybrids. Sequences for the *trnL-F* region of plastid DNA indicate that *S. torminalis* was the female parent of both hybrids. As the two hybrids thus appear to result from hybridisation between the same parents and the hybridisation took place in the same direction, we include the unknown hybrid within *S. × vagensis*.

KEYWORDS: AFLP, *Sorbus aria*, *S. rupicola*, *S. torminalis*, *S. × vagensis*, *trnL-F* region sequencing, Whitebeams.

### INTRODUCTION

In the British Isles, the *Sorbus latifolia* (Lam.) Pers. (Broad-leaved Whitebeam) aggregate contains six taxa which are generally thought to have arisen by hybridisation between *S. torminalis* (L.) Crantz (Wild Service-tree) and members of the *S. aria* (L.) Crantz aggregate (Common Whitebeam) (Sell 1989).

On 8 September 1996, M. Kitchen, C. Kitchen and N. J. Wray found an unknown member of the *S. latifolia* group on remote, inaccessible cliffs of the River Severn at Naas in West Gloucester (v.c. 34). About 13 distinct plants, ranging in size from small suckers/saplings to moderate-sized trees, were present in an area of steep, eroding cliff in an area of about 30 m × 10 m, mixed with *S. torminalis*, and about 50 m from *S. rupicola*. The plants were morphologically uniform, and fruiting sparsely. They occurred on Old Red Sandstone rocks above the River Severn, 2–10 m above the mean tide line. This wood is very rich in woody species, and supports *Acer campestre*, *A. pseudoplatanus*, *Corylus avellana*, *Crataegus monogyna*, *Fagus sylvatica*, *Fraxinus excelsior*, *Ilex aquifolium*, *Ligustrum vulgare*, *Prunus domestica*, *P. spinosa*, *Pyrus communis*, *Quercus petraea*, *Q. robur*, *Rosa canina* s.s. and *R. arvensis*, and is W8 *Fraxinus excelsior-Acer campestre-Mercurialis perennis* woodland (Rodwell 1991). These plants were probably first found by the late S. H. Bishop in 1982 (BRISTM).

As the unknown *Sorbus* was growing with *S. torminalis* and *S. rupicola* in the supposed absence of *S. aria*, it was suggested to be the hybrid between them (Kitchen & Kitchen 1997, 2000). On 18 September 1999, M. and C. Kitchen showed T. Rich the trees, which differed markedly in leaf morphology from other members of the *S. latifolia* group (cf. illustrations in Rich & Jermy 1998) and did not clearly match the descriptions in Warburg (1962) or Sell (1989). The leaves were nearly simple to weakly lobed, and the fruits were 12–14 mm long  $\times$  10–13 mm wide, brownish-orange with numerous small lenticels, and had well-formed seeds to 4.5 mm long. The weakly lobed leaves and large fruits might be characters expected if *S. rupicola* was the simple-leaved parent, and thus the suggested parentage looked plausible. However, the discovery of *Sorbus aria* (L.) Crantz nearby during the 1999 visit suggested that it might be the simple-leaved parent, as its primary diploid hybrid with *S. torminalis* (= *S.  $\times$  vagensis* Wilmott) is widespread in the nearby Wye Valley.

If the parentage of the unknown hybrid could be confirmed as *S. rupicola*  $\times$  *S. torminalis*, this would be of significant interest as previous reports of this hybrid refer to other taxa which are now treated as distinct species, and these plants might therefore also merit specific rank. The widely-cited records for *S. rupicola*  $\times$  *S. torminalis* from v.c. 41 Glamorgan (Cefn Coed) and v.c. 42 Brecon (Dan-y-Craig) (Druce 1917; Ley 1901; Marshall 1916; Richards 1975; Stace 1997) refer to *S. leyana* Wilmott (which is not of this parentage; Lemche 1999) and both sites are actually in v.c. 42 Brecon. Wilmott (1934) suggested that *S. subcuneata* Wilmott had arisen from *S. rupicola* and *S. torminalis* (or possibly what is now known as *S. devoniensis*), and Sell (1989) also regarded this as possible parentage. Alternatively, if the simple-leaved parent was *S. aria*, then the Naas taxon could be included in *S.  $\times$  vagensis*. It was therefore decided to investigate the identity of the trees in more detail.

The taxa were investigated using comparative morphology and a combination of amplified fragment length polymorphism (AFLP<sup>TM</sup>) fingerprinting and DNA sequencing. Only limited sampling for DNA analysis was carried out as the methods were also being used as a trial to see if they could be applied to the genus as a whole, extending the work of Lemche (1999).

AFLP is a highly reproducible method of obtaining genetic fingerprints from small amounts of DNA consisting of DNA fragments of different sizes (number of base pairs) which are then visualised as bands on a gel (Vos *et al.* 1995). It has been used in studies within and between populations (e.g. Qamaruz-Zaman *et al.* 1998; Fay *et al.* 1999, 2000) and for investigation of the origin of taxa of hybrid origin (e.g. Beismann *et al.* 1997; Fay 1998).

Plastid and mitochondrial DNA are normally inherited maternally (e.g. Corriveau & Coleman 1988) and sequencing loci from the plastid genome can therefore provide information about the maternal parent in hybrids. The mode of plastid inheritance is not known in *Sorbus*, but Corriveau & Coleman (1988) showed it to be maternal rather than biparental in *Pyracantha* and *Rosa*, and this is therefore the likely scenario in *Sorbus*. Many plastid regions can be sequenced using universal primers, and for this project we chose to use the *trnL-F* region (the *trnL* intron and the *trnL-F* intergenic spacer; Taberlet *et al.* 1991) as it has been widely used at Kew and elsewhere and has been shown to be a relatively straightforward locus to sequence.

It might also be possible to indicate the parentage cytologically as *S. aria* and *S. torminalis* are diploid ( $2n=34$ ) and *S. rupicola* tetraploid ( $2n=68$ ); a *S. torminalis* hybrid with the latter might thus be expected to be triploid. One root cutting was taken but failed to establish.

## METHODS

### MORPHOLOGY

Leaf morphology was measured on fresh material from Naas Cliff and the Wye Valley, and from existing dried specimens in NMW from the Gloucestershire/Herefordshire/Monmouthshire/Brecon/Somerset area. Thirty leaves from lateral rosettes in well-lit situations were selected for *S. torminalis*, *S. rupicola*, *S.  $\times$  vagensis* and the unknown hybrid. Sixty leaves of *S. aria* were selected as it is more variable. The length (excluding petiole) and width of each leaf were measured, and the number of pairs of veins counted. The length:width ratio was then calculated and plotted against the number of veins as averages for the taxa. This graphical method has been found to be useful for distinguishing between *S. aria* (broad leaves with many veins) and *S. rupicola* (narrow leaves with few veins) elsewhere (Rich, unpublished), and might also therefore indicate which is the parent of the hybrid.

TABLE 1. COLLECTION INFORMATION FOR PLANT MATERIAL USED IN THE DNA ANALYSIS

Species/hybrid	Locality	NMW accession no.	Kew DNA Bank No.
<i>Sorbus aria</i>	River Severn, Naas, v.c. 34	V.2000.009.103	10321
<i>Sorbus aria</i>	Coldwell Rocks, v.c. 34	V.2000.009.104	10322
<i>Sorbus aria</i>	Lords Wood, v.c. 36	V.2000.009.105	10323
<i>Sorbus rupicola</i>	River Severn, Naas, v.c. 34	V.2000.009.106	10319
<i>Sorbus rupicola</i>	Coldwell Rocks, v.c. 34	V.2000.009.107	10320
<i>Sorbus torminalis</i>	River Severn, Naas, v.c. 34	V.2000.009.108	10324
<i>Sorbus torminalis</i>	River Severn, Naas, v.c. 34	V.2000.009.109	10325
<i>Sorbus torminalis</i>	Coldwell Rocks, v.c. 34	V.2000.009.110	10326
<i>Sorbus</i> × <i>vagensis</i>	Coldwell Rocks, v.c. 34	V.2000.009.111	10327
<i>Sorbus</i> × <i>vagensis</i>	Lords Wood, v.c. 36	V.2000.009.112	10328
<i>Sorbus</i> hybrid	River Severn, Naas, v.c. 34	V.2000.009.113	10317
<i>Sorbus</i> hybrid	River Severn, Naas, v.c. 34	V.2000.009.114	10318

## DNA EXTRACTION

Collections of leaves were made from *S. aria*, *S. rupicola*, *S. torminalis*, *S. × vagensis* and the unknown hybrid from Naas Cliff and Coldwell Rocks, Gloucestershire, and Lords Wood, Doward, Herefordshire on 30 June 2000 by T. Rich. Two leaves from each tree were torn into narrow strips and put straight into desiccant silica gel in which they were kept until processed (Chase & Hills 1991). Voucher specimens were collected at the same time for NMW and K. The 12 samples included in the DNA analysis are listed in Table 1 with their origin, cross-referenced with the NMW accession numbers and the Kew DNA bank numbers.

DNA was extracted from approximately 0.2 g of dried material using a modified 2×CTAB (cetyltrimethyl-ammonium bromide) procedure (Doyle & Doyle 1987), purified and quantified using a spectrophotometer.

## AFLP FINGERPRINTING

AFLPs were performed according to the AFLP Plant Mapping Protocol of PE Applied Biosystems Inc. (ABI). For each specimen, 0.5 µg of DNA was digested using the restriction enzymes MseI and EcoRI. Standard adaptors (PE Applied Biosystems) were ligated to the genomic digests. Twenty-four unique primer combinations were screened for their ability to amplify scoreable bands, from which two were selected for this study. Amplification reactions were separated on a 5% polyacrylamide gel using an ABI 377 automated sequencer. Genescan 2.1 and Genotyper 2.0 were used to analyse bands. Bands were scored as either present (1) or absent (0) for all individuals and exported as a binary matrix which was analysed using the UPGMA (Unweighted Pair-Group Method using Arithmetic Averages) algorithm in the software package PAUP version 4.0d64 for Macintosh (Swofford 1998) and by principle co-ordinates analysis (PCOA) in the R Package for Multivariate Analysis version 4.0 (Casgrain & Legendre 1999) using Jaccard's coefficient (Jaccard 1908).

*trnL-F* REGION SEQUENCING

Amplification of the *trnL-F* region from total DNA was carried out using the primers c and f of Taberlet *et al.* (1991). Modified dideoxy cycle sequencing with dye terminators run on an ABI 377 automated sequencer were used to sequence the purified amplification products directly. Both strands were sequenced for 75–100% of the *trnL-F* region, again with the c and f primers. Internal primers were not required. *Sorbus domestica* was used as an outgroup for comparative purposes.

## RESULTS

## MORPHOLOGY

Illustrations of typical sun leaves from lateral rosettes from Naas Cliff are shown in Figure 1 with material from other sources in the region to show the variation. The Naas Cliff plants show similarities to *S. × vagensis*, but the leaves are generally only weakly lobed.

The plot of leaf characters shows that morphologically the unknown hybrid fell close to *S. × vagensis* and between *S. aria* and *S. torminalis*, not between *S. rupicola* and *S. torminalis* (Figure 2).

## AFLP FINGERPRINTING

Two primer combinations, B10 (MseI-CAC and EcoRI-ACT) and G21 (MseI-CTA and EcoRI-AGG), were selected for use in this study on the basis of the lack of background “noise” and the presence of variation between the taxa being studied. Specimen traces showing the general high degree of similarity are shown in Figure 3.

The binary matrix consisted of 109 characters, 45 being bands with primer combination B10 and 64 being bands with G21. Of these, 25 were found in all individuals. Thirty of the 84 polymorphic bands came from B10 and 54 from G21. The data are summarised in Table 2. Apart from the non-variable bands, the commonest types of band were those found in *S. torminalis* + *S. × vagensis* + the unknown hybrid, those found in *S. aria* + *S. rupicola* + *S. × vagensis* + the unknown hybrid, and those unique to *S. rupicola*. Other classes were relatively infrequent. No bands were found that were shared between *S. rupicola* and *S. × vagensis* and/or the unknown hybrid which were not also found in *S. aria* or *S. torminalis*.

In both UPGMA (Figure 4) and PCOA (Figure 5), all species and hybrids were distinguishable. *Sorbus aria* and *S. rupicola* fell close to each other, *S. torminalis* was the most distant from this species pair, and *S. × vagensis* and the unknown hybrid fell in intermediate positions.

The three individuals of *S. aria* had 49, 52 and 53 bands (average 51). The two individuals of *S. rupicola* each had 67 bands, and each had one unique band. Given the high degree of similarity between the two individuals, it is possible that these two bands are homologous, i.e. they represent alleles of the same locus, but it would require studies of how the bands segregate to confirm this.

TABLE 2. DISTRIBUTION OF BANDS AND THEIR RELATIVE FREQUENCIES. T, PRESENT IN *SORBUS TORMINALIS*. V, PRESENT IN *S. × VAGENSIS*. K, PRESENT IN UNKNOWN HYBRID. A, PRESENT IN *S. ARIA*. R, PRESENT IN *S. RUPICOLA*

Type of band	Number	Percentage
T+V+K+A+R	25	23
T+V+K	20	18
V+K+A+R	18	17
R	12	11
T+V	6	6
K+A+R	6	6
T+V+K+R	5	5
T	5	5
A	3	3
T+K	3	3
T+V+A+R	1	1
K	1	1
V+K+A	1	1
K+A	1	1
T+A	1	1
V+A+R	1	1

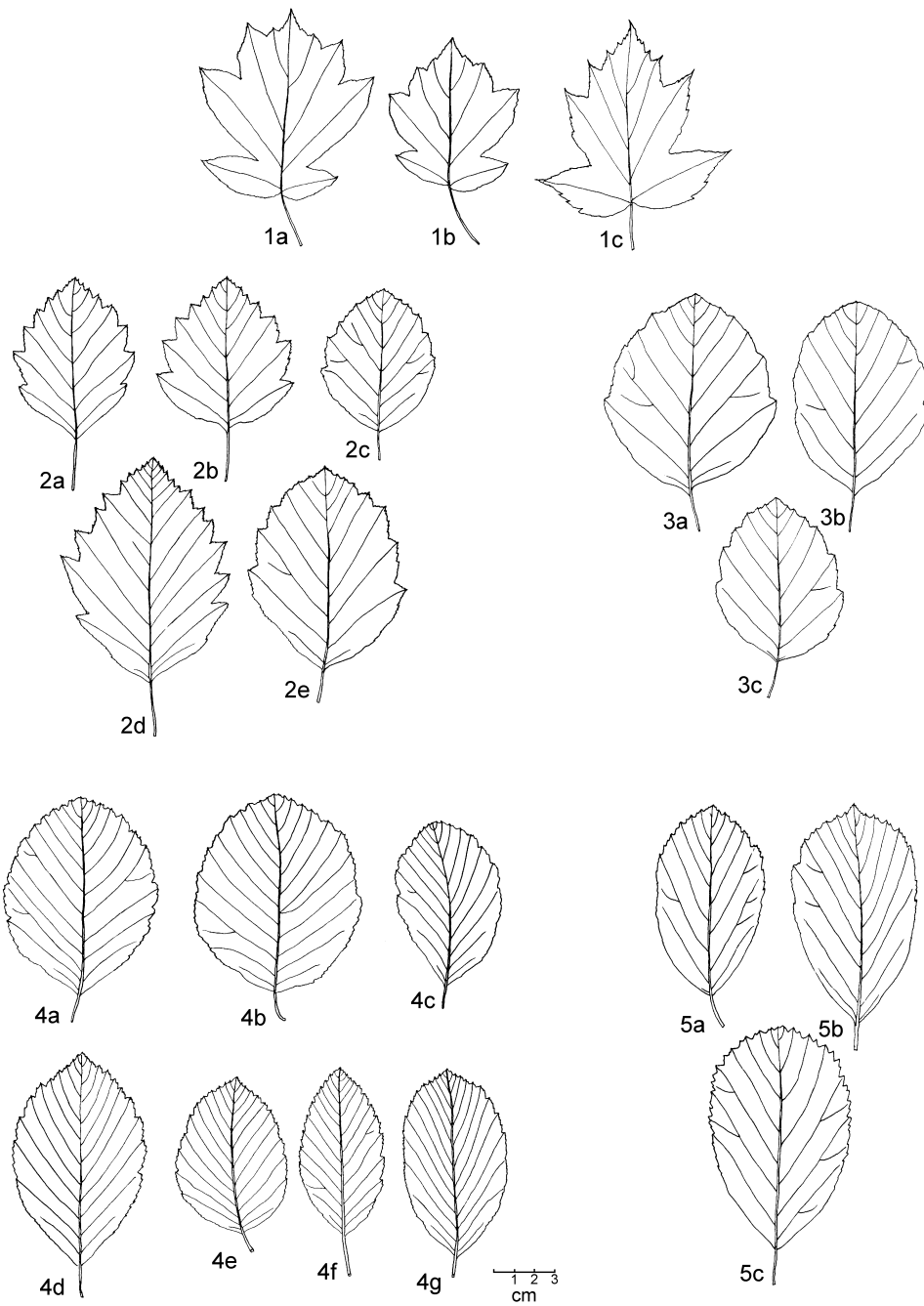


FIGURE 1. Silhouettes of *Sorbus* taxa studied in morphological analysis. 1. *S. torminalis*. a, b, Naas Cliff, Gloucestershire. c, Coldwell Rocks, Gloucestershire. 2. *S. × vagensis*. a, Coldwell Rocks. b, c, Symonds Yat, Gloucestershire. d, Cheddar Wood, Somerset. e, Wyndcliff, Monmouthshire. 3a-c. Unknown *Sorbus* hybrid, Naas Cliff. 4. *S. aria*. a, b, Naas Cliff. c, d, Coldwell Rocks, e-g, Avon Gorge, Gloucestershire. 5. *S. rupicola*. a, b, Naas Cliff. c, Coldwell Rocks.

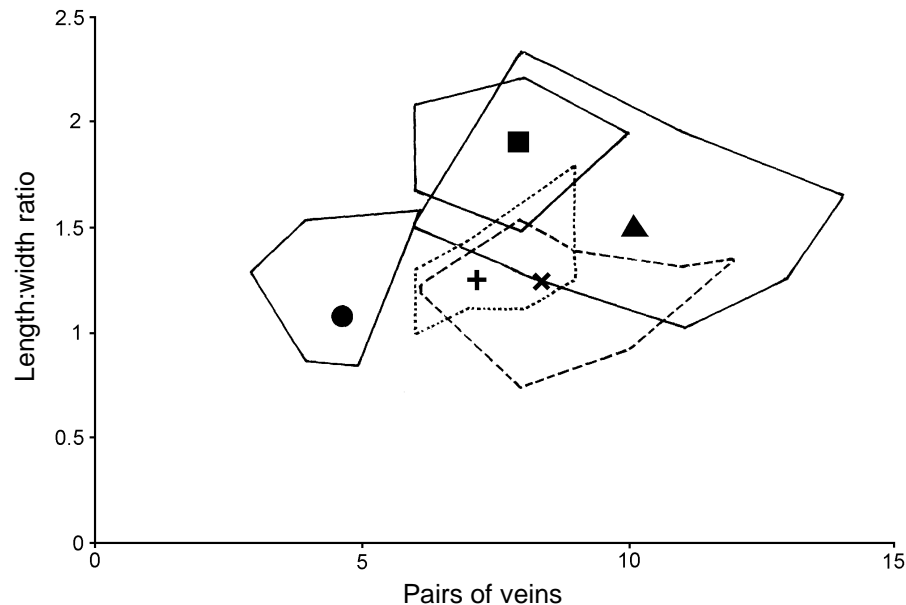


FIGURE 2. Number of veins against leaf length:width ratio for *Sorbus* taxa from NMW collections. Plots show polygons covering all the variation with the means at the approximate centres.  $\blacktriangle$  = *S. aria* (n=60).  $\blacksquare$  = *S. rupicola* (n=30).  $\bullet$  = *S. torminalis* (n=30).  $\times$  and - - - = *S. \times vagensis* (n=30).  $+$  and  $\bullet\bullet$  = Unknown hybrid (n=30).

The three exemplars of *S. torminalis* had 58, 58 and 61 bands (average 59). The *S. \times vagensis* samples had 73 and 74 bands and they differed from each other in the presence or absence of five bands. The two exemplars of the unknown hybrid fell slightly separate from *S. \times vagensis* in both analyses. They had 77 and 79 bands (average 78), and they differed overall by two bands.

In the PCOA, the first three co-ordinates accounted for 61, 14 and 9% (total 84%) respectively, of the variation, and thus the signal in the data is strong, with all other co-ordinates only accounting for 16% of the variation. Figure 5 shows co-ordinate 1 plotted against co-ordinate 2. The result of plotting co-ordinate 1 against co-ordinate 3 was very similar, and therefore is not presented.

#### *trnL-F* REGION SEQUENCING

The aligned *trnL-F* sequences gave a matrix of 618 characters. Of these, 608 were constant, and 617 were constant in all taxa apart from *S. domestica*. The remaining sequences were of two types. *Sorbus torminalis*, *S. \times vagensis* and the unknown hybrid had identical sequences of the 'torminalis' type, and *S. aria* and *S. rupicola* had identical sequences of the 'aria' type. In addition to differing from each other by a single substitution (G in 'aria' and T in 'torminalis' at base 406 in the aligned matrix), the 'aria' type also had a single base insertion relative to the 'torminalis' type (an A at base 405).

#### DISCUSSION

The morphology and AFLP fingerprinting indicate that the likely parentage of the unknown Naas Cliff hybrid is *S. aria*  $\times$  *S. torminalis*, rather than *S. rupicola*  $\times$  *S. torminalis*.

In addition to falling separately from the species in the AFLP analyses, *S. \times vagensis* and the unknown hybrid also showed clear additivity between the genotypes of *S. aria* and *S. torminalis*, with both apparently having received 18 bands from *S. aria* and 20 bands from *S. torminalis*. On the basis of the sampling here, *S. torminalis* is confirmed as one parent of both *S. \times vagensis* and the unknown hybrid. The evidence for *S. aria* as the other parent is not so clear, and what evidence

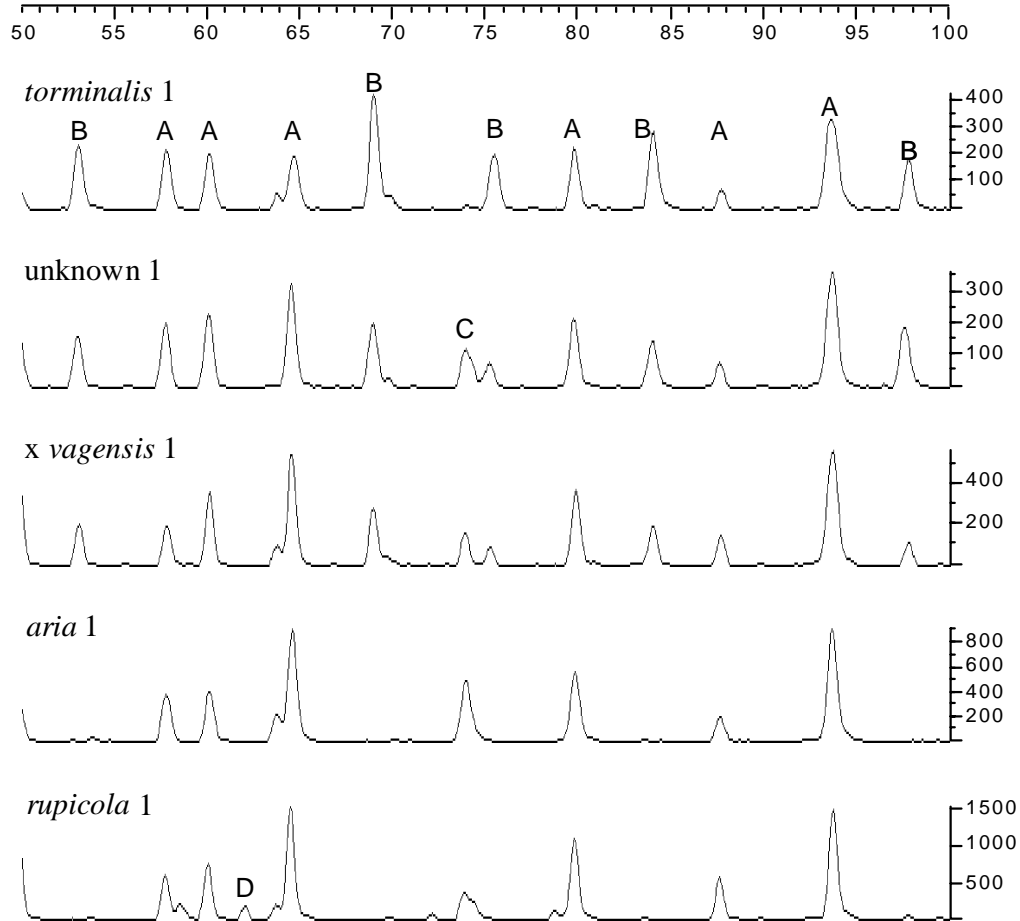


FIGURE 3. Specimen trace for samples of *Sorbus* taxa, showing a general high degree of similarity. Examples of different types of band are indicated by different letters. A = universal bands. B = bands present in *S. torminalis* + hybrids. C = bands present in *S. aria*/*S. rupicola* + hybrids. D = bands only present in *S. rupicola*. Numbers at the top of the figure indicate the sizes of the fragments in base pairs and numbers at the side indicate the strength of peaks in arbitrary units of fluorescence.

there is excludes *S. rupicola* rather than proving *S. aria* to be the other parent. *S. rupicola* has twelve unique bands (compared to three in *S. aria* and five in *S. torminalis*), and no bands are shared by *S. rupicola* and either *S. × vagensis* or the unknown hybrid which are not also present in either *S. aria* or *S. torminalis*. There is thus strong evidence to exclude *S. rupicola* as a parent in both cases, and both *S. × vagensis* and the unknown hybrid appear to be the result of hybridisation between *S. aria* and *S. torminalis*. As would be expected of hybrids, *S. × vagensis* and the unknown hybrid both have more bands than either parent as a result of additivity.

The sequence analysis showed that both *S. × vagensis* and the unknown hybrid share the plastid type of *S. torminalis*. Thus *S. torminalis* was apparently the female parent in both cases. As both *S. × vagensis* and the unknown hybrid appear to have the same two parental species and the hybridisation took place in the same direction, we include the unknown Naas hybrid in *S. × vagensis*. Hybridisation has thus occurred independently at Naas and Coldwell Rocks, and the morphological variation observed in *S. × vagensis* (cf. Sell 1989, and Figures 1 and 2) may thus be partly explained in terms of multiple hybridisation events. What is surprising, given the large geographical overlap in range exhibited by the parents in Britain, is the apparent limitation in the

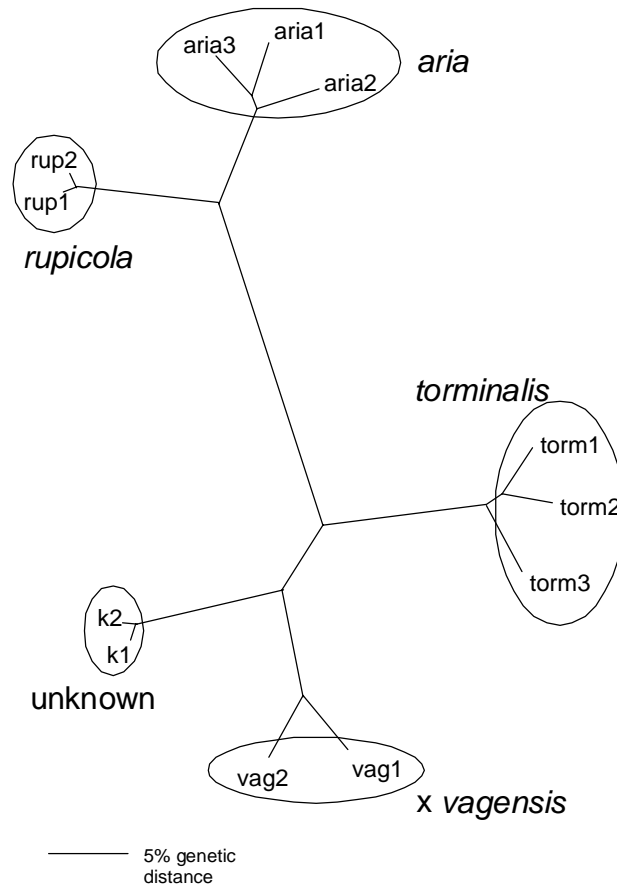


FIGURE 4. UPGMA dendrogram showing relationships of *Sorbus* taxa whose DNA was studied.

occurrence of *S. × vagensis* to the region around the Severn Estuary. Sell (1989) explains this in terms of ecological separation, the Severn Estuary/Wye valley area being one of the few places where the parents grow close together.

The DNA methods were found to be useful tools for analysing the species and hybrids in the group, and show great potential for further work. The high number of bands shared between *S. aria* and *S. rupicola* (51 out of a possible 53) and the higher number of bands present in *S. rupicola* suggest that *S. rupicola* may also be of ancient allopolyploid hybrid origin, with *S. aria* s.s. as one parent and another member of *S. aria* s.l. as the other. Further sampling of both taxa and of other members of the *S. aria* group would be required before a conclusive answer as to its parentage can be obtained.

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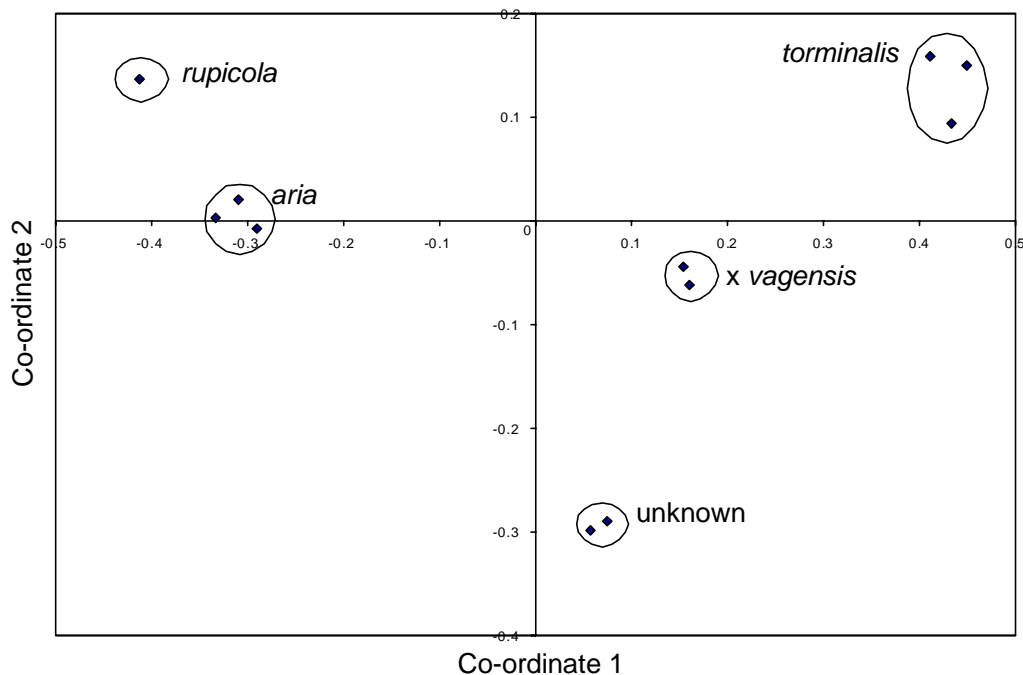


FIGURE 5. Plot of the first and second axes from a PCO analysis of *Sorbus* taxa whose DNA was studied. The two samples of *S. rupicola* have identical co-ordinates.

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