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Chloroplast diversity in the genus *Malus*: new insights into the relationship between the European wild apple (*Malus sylvestris* (L.) Mill.) and the domesticated apple (*Malus domestica* Borkh.)

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Abstract

To unravel the relationship between the European wild apple, *Malus sylvestris* (L.) Mill., and its domesticated relative *M. domestica* Borkh., we studied chloroplast DNA variation in 634 wild and 422 domesticated accessions originating from different regions. Hybridization between *M. sylvestris* and *M. domestica* was checked using 10 nuclear microsatellites and a Bayesian assignment approach. This allowed us to identify hybrids and feral plants escaped from cultivation. Sixty-eight genotypes belonging to 12 other wild *Malus* species, including 20 *M. sieversii* (Ledeb.) Roem. accessions were also included in the analysis of chloroplast diversity. Marker techniques were developed to type a formerly described duplication and a newly detected transversion in the *mat*K gene. Chloroplast DNA variation was further investigated using PCR-RFLP (Polymerase Chain Reaction-Random Fragment Length Polymorphism), and haplotypes were constructed based on all mutational combinations. A closer relationship than presently accepted between *M. sylvestris* and *M. domestica* was established at the cytoplasmic level, with the detection of eight chloroplast haplotypes shared by both species. Hybridization between *M. sylvestris* and *M. domestica* was also apparent at the local level with sharing of rare haplotypes among local cultivars and sympatric wild trees. Indications of the use of wild *Malus* genotypes in the (local) cultivation process of *M. domestica* and cytoplasmic introgression of chloroplast haplotypes into *M. sylvestris* from the domesticated apple were found. Only one of the *M. sieversii* trees studied displayed one of the three main chloroplast haplotypes shared by *M. sylvestris* and *M. domestica*. This is surprising as *M. sieversii* has formerly been described as the main maternal progenitor of the domesticated apple. This study hereby reopens the exciting discussion on the origin of *M. domestica*.

Keywords: chloroplast DNA variation, domestication, hybridization, *Malus domestica*, *Malus sylvestris*, *mat*K

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Introduction

Hybridization and introgression have probably played important roles in the evolution of the Rosaceae (Phipps *et al*. 1990; Katayama & Uematsu 2003). This has caused complex taxonomic relationships within this family, which are further complicated by the cultivation of Rosaceous species for their fruits or as ornamentals (Morgan *et al*. 1994). Despite its economic importance and wide geographical distribution, the origin of the domesticated apple (*Malus domestica* Borkh.), the most important Maloideae fruit crop, remains unclear. The currently most widely accepted theory, based mainly on morphological and molecular evidence, points to series *Malus* (Dunemann *et al*.

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1994; Forsline *et al*. 1994; Morgan *et al*. 1994; Savolainen *et al*. 1995) and specifically towards *M. sieversii* (Ledeb.) Roem., a wild species of Central Asia, as the most likely maternal ancestor (Robinson *et al*. 2001; Forte *et al*. 2002; Harris *et al*. 2002). This latter conclusion is mainly based on the presence of large fruits and the occurrence of duplications at specific positions in the chloroplast *mat*K gene in *M. sieversii* (Robinson *et al*. 2001). Duplication I was found in most species of the section *Malus* and was therefore not useful to further analyse the relatedness between these *Malus* species and the domesticated apple. Duplication II was detected in eight out of the nine *M. domestica* trees included in the study and absent in all other species studied, except in one *M. sieversii* accession originating from the Tien Shan region near the Kazakhstan border, in Central Asia. This was interpreted as a confirmation of the maternal contribution of *M. sieversii* to the origin of the domesticated apple (Harris *et al*. 2002). Furthermore, as duplication II was absent in the two *M. sylvestris* genotypes analysed, it was suggested that *M. sylvestris*, the wild apple native to Western Europe, might have contributed little or even nothing to the domesticated apple gene pool (Robinson *et al*. 2001), at least as maternal ancestor. However, as the authors stated, their study was based on a small number of accessions and more domesticated apples should be analysed to ensure that rare hybridization events with other *Malus* species that might have contributed to the early domestication of the apple had not been overlooked. Furthermore, the postulated close relationship between *M. sieversii* and *M. domestica* should be confirmed using additional molecular evidence.

In addition to the purely academic interest in the origin of the domesticated apple, studies on the phylogenetic relationships between the domesticated apple and its wild relatives are relevant as they may point towards taxa that can serve as sources of novel genes for breeding purposes (Robinson *et al*. 2001). Nature conservation issues have also raised interest in the phylogenetic relationship between the European wild apple species *M. sylvestris* and the domesticated apple. *M. sylvestris* has become an endangered tree species in Europe and the remaining individuals are very scattered (Stephan *et al*. 2003). Therefore, the construction of gene banks that can be used as new interbreeding populations is needed for its future conservation. This necessitates the discrimination between 'genuine' wild, hybridized and domesticated apple trees in order to construct gene banks of 'pure' wild genotypes with no signs of introgression of *M. domestica*. This discrimination is not always possible on morphological grounds. Our previous work on the relationship between *M. sylvestris* and *M. domestica*, based on nuclear DNA markers [Amplified Fragment Length Polymorphism (AFLP) and microsatellites], revealed that in Belgium, both species can be clearly discriminated (Coart *et al*. 2003). However, historical introgression events are a major problem when studying hybridization of sympatric species with unlinked nuclear markers since advanced backcrosses will only retain a limited number of alleles from the ancestral species (Pritchard *et al*. 2000; Beaumont *et al*. 2001). The combination of data from nuclear (nDNA) and uniparentally inherited cytoplasmic genetic markers can provide important clues about past and recent hybridizations and thereby on the origins of domesticated plants, as has been shown for example in *Citrus* (Moore 2001) and in potatoes (Hosaka 1995). Duplication II in the *mat*K gene seemed initially to be a promising tool for the study of hybridization between *M. sylvestris* and *M. domestica*.

The main research questions addressed in this study were:

1 Are *M. sylvestris* and *M. domestica* as clearly differentiated at the chloroplast DNA level as at the nuclear DNA level?
2 Could *M. sylvestris* have been a maternal progenitor of *M. domestica*?
3 Is the relationship between *M. sieversii* and *M. domestica* as close as currently assumed?

The samples analysed comprise 1133 genotypes and include *M. sylvestris* genotypes from different geographical regions, old and modern *M. domestica* cultivars, and 12 other *Malus* species. We first typed all these samples with 10 nuclear SSR markers and applied a Bayesian approach (STRUCTURE, Pritchard *et al*. 2000) to discriminate between wild, hybridized and cultivated genotypes using the methodology described previously (Coart *et al*. 2003). We then amplified and sequenced the chloroplast *mat*K region described by Robinson *et al*. (2001) in a set of *M. sylvestris* and *M. domestica* genotypes. When the first results of the sequencing analysis showed that the distribution of duplication II and a newly detected transversion in the *mat*K sequence needed further analysis, we developed simple Polymerase Chain Reaction-based (PCR-based) assays to visualize easily these polymorphisms in the *mat*K gene in the complete sample of genotypes. To characterize other polymorphisms in the chloroplast DNA (cpDNA) of the investigated *Malus* trees, we amplified parts of the cpDNA genome using conserved primers (Demesure *et al*. 1995; Dumolin-Lapègue *et al*. 1997) and digested the amplicons with different restriction enzymes, to generate polymorphic PCR-RFLP markers.

Materials and methods

Supplementary information on the origin of the plant materials used in this study, protocols for DNA extraction and other laboratory techniques are provided in the Supplementary material.

Plant material

In total, 634 genotypes originally labelled as *M. sylvestris* were analysed (Table 1). The majority of the trees (496) were collected in Belgian forests. Individuals from other European regions were also included to investigate intraspecific variation: 76 trees from Denmark, 28 trees from France, 14 from Germany and 20 from Central Asia (obtained from USDA-ARS at Cornell University, Geneva, USA; the accession numbers of the genotypes analysed are provided in the Supplementary material).

For *M. domestica*, 422 trees from different collections were analysed (Table 1). Samples from two Belgian collections of old cultivars were studied: 149 accessions from the collection of the Centre for Agricultural Research (CRA-W, www.cra.wallonie.be/english/dep3/index.html) in Gembloux and 139 accessions from the collection of the National Orchard Foundation (NBS, www.boomgaardenstichting.be/). Cultivars of these collections for which origin is documented date back from the 16th to the 19th century. We also analysed 19 genotypes from a collection of old Danish cultivars (between 100 and 250 years old, The Pometum RVAU, www.pometet.kvl.dk/Engelsk_forside.htm/) and 86 genotypes from the collection of modern apple cultivars of the Centre for Fruit Culture from the Catholic University of Leuven (www.agr.kuleuven.ac.be/dtp/fruit/English_Version.htm), Belgium. The modern cultivars analysed originate from different selection programmes worldwide and represent apple-breeding efforts of the last 100 years and also include older cultivars that are still commercially important or are currently used in breeding programmes. Finally, 25 cider cultivars from the CRA-W collection (originally of French and British origin) were also analysed.

We obtained 68 grafts of 12 additional *Malus* species from the collection of USDA-ARS at Cornell University, Geneva, USA. USDA-ARS genebank accessions codes are provided online and can be accessed directly on the USDA website (www.ars-grin.gov/cgi-bin/npgs/html/) for further information on the exact collection site of the *Malus* genotypes. The 20 *M. sieversii* accessions originate from Kazakhstan, Kyrgyzstan, Uzbekistan, Tajikistan and Russia. Nomenclature of all *Malus* species follows Phipps *et al*. (1990).

Typing of 10 SSR loci

All *M. sylvestris*, *M. domestica* and *M. sieversii* accessions listed in Table 1 were typed for 10 SSR loci, spread over different chromosomes: NZ02b01, NZ04h11, NZ05g08, NZ23g04, NZ28f04, CH01h10, CH01f02, CH01h01, CH02b12 and CH02c06. Normalized nomenclature of the loci follows Liebhard *et al*. 2003, with loci with prefix 'NZ' from Guilford *et al*. (1997) and loci with prefix 'CH' from Gianfranceschi *et al*. (1998). Information on loci NZ02b01, NZ04h11, NZ05g08 and NZ23g04 was kindly provided by J. Keulemans and L. Vanwynsberghe from the Fruit Breeding Centre (FTC) of the University of Leuven. The choice of these 10 SSR loci was based on degree of polymorphism and applicability over different *Malus* species. Amplification and PAGE (Polyacrylamide Gel Electrophoresis) conditions were as described in Coart *et al*. (2003).

The software STRUCTURE (Pritchard *et al*. 2000) with the admixture model was applied on the multilocus SSR data of *M. sylvestris, M. domestica* and *M. sieversii* accessions to infer the different gene pools in the data set. The software implements a model-based Bayesian clustering approach for probabilistic assignment of individuals to a number of clusters with simultaneous estimation of the unknown allele frequencies within them. Assignment of individuals and inference of allele frequencies is performed so that departures from Hardy–Weinberg and gametic-phase disequilibrium within the clusters are minimized. For the identification of hybrids among *M. sylvestris* genotypes, STRUCTURE was applied only on *M. sylvestris* and *M. domestica* accessions. Cultivated genotypes were this time used as learning samples ('population information model', Pritchard *et al*. 2000) and for wild samples, the admixture model was applied. The number of clusters was set at 2 (as in Beaumont *et al*. 2001). This allowed the identification of hybrids between inferred gene pools and the detection of genotypes that were outliers in their sample of origin and that in fact belonged to another gene pool. For the analysis of chloroplast haplotypes, 'pure' *M. sylvestris* genotypes were treated separately from hybrids and feral cultivars (see below).

Using the software SPAGEDI 1.0 (Hardy & Vekemans 2002), differentiation values (F_{ST}) between gene pools were calculated. The significance of the genetic differentiation between groups was tested by comparison of the observed F_{ST} with a distribution of F_{ST} obtained by means of 1000 random permutations of individuals across groups under the null hypothesis of no genetic structure.

*Sequencing of the mat*K *region*

In a first step, 1740 bp of the *mat*K region were amplified and sequenced for nine *M. sylvestris* and five *M. domestica* genotypes. The *mat*K gene was PCR amplified as described in Robinson *et al*. (2001) using primers developed by Sang *et al*. (1997) and Dendauw *et al*. (2002). Primer sequences and reaction conditions are provided as online Supplementary material.

Over the 14 genotypes analysed 1720 bp of the *mat*K gene were invariant. As expected, duplication I was present in all 14 genotypes. Of the 20 variable positions, 18 corresponded to duplication II, which was present in six genotypes and absent in eight. The two other variable positions were at positions 338 and 1027, as shown on the sequence alignment (Fig. 1). As reported by Robinson *et al*. (2001), those genotypes in which duplication II was present

Table 1 Description of the *Malus sylvestris* and *Malus domestica* accessions analysed. 'Traditional cultivar' refers to cultivars which were released before 1900. 'Modern cultivar' refers to cultivars which were released after 1900 or are still commonly used in current apple breeding or production. FL, Flanders (north of Belgium); WL, Wallony (south of Belgium)

Species/origin	Nb*	Provider	Description
Malus sylvestris	634 (542, 71, 21)		
Belgium (FL)	229 (207, 18, 4)	Institute for Forestry and Game Management (IBW)	Forests (Flemish region)
Belgium (WL)	267 (225, 30, 12)	Centre de Recherches Agronomique/Centre de Recherche de la Nature, des Forets et du Bois (CRNFB)	Forests (Walloon region)
Germany	14 (9, 4, 1)	Niedersachsische Forstliche Versuchsanstalt/ LöBF Forstgenbank	Gene banks of federal districts Lower Saxony and Schleswig Holstein
France	28 (19, 7, 2)	Université de Metz	Forests
Denmark	76 (73, 3, 0)	Forest and Landscape Denmark, RVAU	Forests
Central Asia†	20 (9, 9, 2)‡	USDA-ARS, Cornell University, USA	Forests
Malus domestica			
Traditional Belgian (CRA)	149	Centre de Recherches Agronomique (CRA-W)	Gene bank, old cultivars
Traditional Belgian (NBS)	139	National Orchard Foundation (NBS)	Gene bank, old cultivars
Modern cultivars	86	University Leuven, Centre for Fruit Culture (FTC)	Gene bank, modern cultivars
France/UK	25	Centre de Recherches Agronomique (CRA-W)	Gene bank, cider cultivars
Traditional Danish	23	The Pometum, RVAU	Gene bank, old cultivars

*Nb of trees sampled. For the *M. sylvestris* individuals, the results of the STRUCTURE assignment based on nuclear SSR markers are shown between brackets (nb of pure individuals, nb of hybrids with cultivars, nb of feral cultivars).
†USDA-ARS accession codes are provided as online supplementary material.
‡Includes hybrids with *M. domestica* and *M. sieversii*.

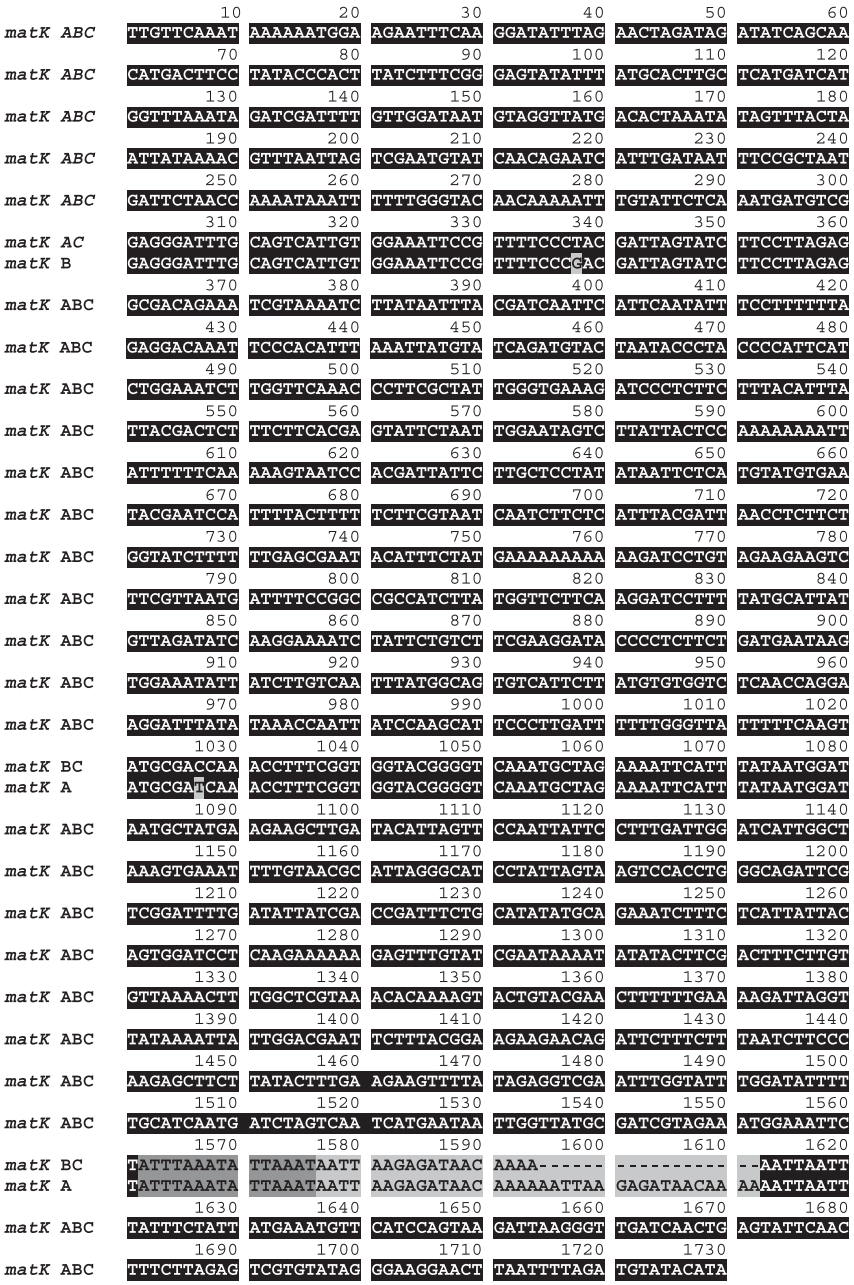

Fig. 1 Sequence of the *mat*K region in *Malus*. Positions where one of the defined alleles (A, B or C, indicated in front of each row) has a deviant base composition are marked in grey. ATTT...: Duplication I; AATT...: Duplication II.

displayed also one T at position 1027, whereas genotypes without duplication II contained a C at this position. This was further confirmed by sequencing this part of the *mat*K gene for 60 additional accessions (30 *M. domestica* and 30 *M. sylvestris*). Among those genotypes in which duplication II was absent, some displayed a G at position 338 and some a T. However, genotypes in which duplication II was present always displayed a T at position 338. This renders a total of three *mat*K alleles in *Malus* (named A, B and C in Fig. 1, EMBL Accession nos AM042561, AM042562 and AM042563, respectively). These three *mat*K alleles can be differentiated by checking for the presence of duplication II and the polymorphism at position 338. Therefore, in a next step simple marker tests were developed to screen the large number of accessions for these two polymorphisms.

Marker development

To detect the presence of duplication II using a simple PCR assay, we developed a new primer '*mat*KdupF' (5'-ATAGAGGTCGAATTTGGTATTTGGAT-3'). The fragment containing duplication II was amplified with this newly developed primer and primer *trn*K2R (see Supplementary material). After PAGE, the fragments were scored as

duplication II present (PCR product 351 bp) or duplication II absent (PCR product 333 bp).

To type the transversion at position 338, a simple assay was developed. The first part of the *mat*K gene was amplified with primers P1F and 1RNL1A (see Supplementary material). When a G is present at position 338, a recognition site for the restriction enzyme Hpy99I is formed. The genotypes were scored after restriction as mutation absent (position 338 is a T, one DNA fragment of 643 bp after restriction of the PCR product) or mutation present (position 338 is a G, one restriction fragment of 338 bp and one of 305 bp).

Haplotyping of the chloroplast genome with PCR-RFLP markers

In a first step, 10 pairs of universal chloroplast primers (trnH/trnK1, trnC/trnD, trnD/trnT, psbC/trnS, trnS/trnfM, psaA/trnS, trnS/trnT, trnM/rbcL, trnT/trnF and trnK1/trnK2) were used to test amplification on 10 randomly chosen *Malus* genotypes belonging to different species. Primer pairs are as described in Dumolin-Lapègue *et al*. (1997) and Lofty *et al*. (2003), which is a new combination derived from Demesure *et al*. (1995). Based on these results, the three primer pairs that yielded clear PCR fragments in the 10 genotypes analysed were selected (trnH/trnK1, trnD/trnT and trnS/trnT). In a second step, the PCR products amplified with these three primer pairs in 48 genotypes from different species were digested with 16 different restriction endonucleases (*Alu*I, *Mbo*I, *Dpn*I, *Mun*I, *Mlu*I, *Mse*I, *Eco*RI, *Hin*fI, *Hin*dIII, *Pvu*II, *Xmi*I, *Xba*I, *Sal*I, *Rsa*I, *Taq*I, *Pst*I). Following restriction, polymorphisms were only revealed in the region trnD/trnT using *Mse*I and *Hin*fI and in the region trnH/trnK1 with *Mse*I, *Hin*dIII, *Eco*RI, *Mun*I and *Pvu*I. Restriction patterns derived for each of these cpDNA regions using different restriction enzymes provided identical information on the 48 genotypes analysed (results not shown); therefore, the enzymes resulting in the clearest restriction patterns were selected to type the complete set of *Malus* accessions: *Mse*I for region trnT/trnD and *Eco*RI for region trnH/trnK1. For further details on experimental procedures, see online Supplementary material.

Combination of marker data into chloroplast haplotypes

All mutational combinations were used to construct overall chloroplast haplotypes. Based on the frequency of chloroplast haplotypes in the different species and origins, total gene diversity (h) and allelic richness (r) were calculated for all samples containing more than three accessions using the software CONTRIB (Petit *et al*. 1998). As the allelic diversity is highly dependent on the sample size (Nei *et al*. 1975), the allelic richness was standardized to cope with uneven sample sizes according to the rarefaction technique (El Mousadik & Petit 1996). Levels of gene diversity were compared using independent-samples t-tests and a Bonferroni adjustment for multiple comparisons.

Finally, *M. sylvestris* and *M. domestica* accessions were grouped according to their chloroplast haplotype. One-way ANOVA and LSD post hoc tests were used to test for significant differences in percentage of assignment to the cultivated gene pool (data obtained by STRUCTURE analysis of nuclear SSR data) among the different haplotypes.

Results

Delineation of gene pools based on nuclear information

The genotypes originally labelled as *M. sylvestris*, all the cultivars and the 20 *M. sieversii* genotypes obtained from the genebank in Geneva were included in this analysis. Based on the likelihood output of the genetic admixture analysis using no prior information on sample origin, the division into three clusters best described the distribution of individuals within the collective sample. STRUCTURE results showed that *Malus domestica* (represented by gene pool 2), *M. sylvestris* (represented by gene pool 3) and *M. sieversii* (represented by gene pool 1) constitute clearly differentiated groups (Table 2). However, a certain proportion of admixture was found.

The only sample of *M. sylvestris* that showed clear signs of admixture with *M. sieversii* (31%) was that composed by trees collected in Central Asia (Table 2). This was a very heterogenous sample as it was also assigned for 20% to the domesticated gene pool, suggesting that this sample is not purely *M. sylvestris* and that many trees in the sample are probably mislabelled. All other *M. sylvestris* samples showed a lower extent of admixture with *M. domestica*, with proportions of their genetic information assigned to the domesticated gene pool ranging from 4% to 17%.

After exclusion of migrants and hybrids (see next section), differentiation among species was calculated and both wild species were shown to be genetically equally distant from *M. domestica*: $F_{ST} = 0.09$ between *M. sieversii* and *M. domestica* and $F_{ST} = 0.11$ between *M. sylvestris* and *M. domestica*. Differentiation between the two wild species *M. sylvestris* and *M. sieversii* was the largest with $F_{ST} = 0.13$. All differentiation values were significant at the 0.001 level.

Identification of hybrids and feral cultivars among M. sylvestris *accessions*

The proportion of assignment of each individual genotype to the domesticated gene pool reveals the presence of both cultivars and hybrids among the accessions sampled as *M. sylvestris* (STRUCTURE analysis using 'learning samples', Fig. 2). We considered genotypes originally labelled as *M. sylvestris* but that were assigned for more than 20% to gene pool 2 (*M. domestica*) as hybrids and those assigned for

Category of origin	Gene pool 1 (*M. sieviersii*)	Gene pool 2 (*M. domestica*)	Gene pool 3 (*M. sylvestris*)
M. sylvestris Belgium (WL)	0.04	0.17	0.79
M. sylvestris Belgium (FL)	0.02	0.08	0.90
M. sylvestris Denmark	0.02	0.04	0.94
M. sylvestris France	0.04	0.12	0.84
M. sylvestris Germany	0.02	0.16	0.82
M. sylvestris Central Asia	0.31	0.20	0.50
Total *M. sylvestris*	0.07	0.13	0.80
M. domestica Belgium (NBS)	0.02	0.91	0.07
M. domestica Belgium (CRA)	0.02	0.91	0.07
M. domestica Denmark	0.15	0.73	0.12
M. domestica modern	0.05	0.89	0.06
M. domestica cider apples	0.08	0.75	0.17
Total *M. domestica*	0.06	0.84	0.10
M. sieversii	0.89	0.06	0.05

Table 2 Summary of the results obtained by the Bayesian assignment procedure based on SSR data (STRUCTURE with 'admixture model'). Figures are the proportions of estimated membership to each of three inferred gene pools for genotypes of a given category of origin. FL, Flanders (north of Belgium); WL, Wallony (south of Belgium)

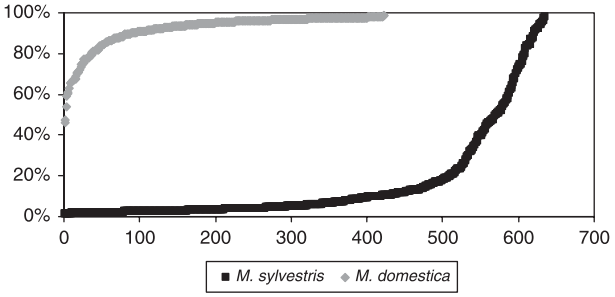

Fig. 2 Results of the STRUCTURE analysis with 'population information model'. For each individual genotype, the proportion of its genotypic information assigned to the domesticated gene pool is shown. The genotypes have been arranged in ascending order of assignment to the domesticated gene pool.

more than 80% to gene pool 2 as feral cultivars. These cut-off values were chosen arbitrarily from the assignment values shown in Fig. 2. By doing so, 92 *M. sylvestris* accessions were identified as hybrid (71 individuals) or as feral *M. domestica* (21 individuals). These 92 deviant genotypes are discussed separately for their chloroplast haplotypes. Only nine of the *M. sylvestris* trees from Central Asia were genuine *M. sylvestris* trees according to this classification.

Variation in matK *region: presence of duplication II and SNP at position 338*

Duplication II, previously suggested to be diagnostic for *M. domestica* (Robinson *et al*. 2001), was present in 24% of the *M. sylvestris* genotypes analysed in this study. Moreover, a significant fraction of the cultivars (41%) did not carry duplication II. Duplication II was also found in one *M. sieversii* accession and three *M. prunifolia* accessions, both belonging to the series *Malus*, but was absent from the remainder of sections and series.

Table 3 Definition of 16 chloroplast haplotypes based on all mutational combinations

cp haplotype	*mat*KdupII*	*mat*K Position 338	HK1-*Eco*RI	DT-*Mse*I
1	0	T	1	1
2	0	T	1	4
3	0	T	1	6
4	0	T	2	1
5	0	T	2	2
6	0	T	2	3
7	0	T	2	4
8	0	G	1	1
9	0	G	2	1
10	0	G	2	2
11	0	G	2	3
12	0	G	2	7
13	0	G	2	8
14	1	T	1	1
15	1	T	1	2
16	0	G	1	2

*0 = duplication II absent, 1 = duplication II present.

On average, 11% of *M. sylvestris* genotypes carried a G at position 338. In old cultivars a G was found more frequently than in modern and cider cultivars. A G at position 338 was further found in other species from series *Malus* (*M. kirghisorum*, *orientalis*, *prunifolia* and *sieversii*), series Baccata (*M. hupehensis*) and section *Sorbomalus* series Kansuenses (*M. fusca* and *M. kansuenses*). It was confirmed that a G at position 338 never occurred in trees containing duplication II.

Combination of marker data into chloroplast haplotypes

When all marker data were combined, 16 different chloroplast haplotypes (cpht) could be defined (Table 3). Table 4

Table 4 Frequency of 16 chloroplast haplotypes across *Malus* species and origins. Last two lines: frequency of chloroplast haplotypes in hybrid genotypes and in feral cultivars sampled in the nature and originally classified as *M. sylvestris*. FL: Flanders (North of Belgium); WL: Wallony (South of Belgium)

Category of origin	Sec/Ser	Nb	Cpht1	Cpht2	Cpht3	Cpht4	Cpht5	Cpht6	Cpht7	Cpht8	Cpht9	Cpht10	Cpht11	Cpht12	Cpht13	Cpht14	Cpht15	Cpht16
M. sylvestris Belgium (WL)	M/M	225	0.604								0.018	0.151	0.018			0.209		
M. sylvestris Belgium (FL)	M/M	207	0.565									0.077				0.348	0.005	0.005
M. sylvestris Denmark	M/M	73	0.918				0.014			0.014		0.014				0.041		
M. sylvestris France	M/M	19	0.842													0.158		
M. sylvestris Germany	M/M	9	1.000															
M. sylvestris Central Asia	M/M	9	0.778													0.222		
Total *M. sylvestris*		542	0.649				0.002			0.002	0.007	0.094	0.007			0.234	0.002	0.002
M. domestica Belgium (NBS)	M/M	139	0.108									0.338	0.007			0.547		
M. domestica Belgium (CRA)	M/M	149	0.081								0.034	0.255	0.007			0.570	0.047	0.007
M. domestica Denmark	M/M	23	0.130			0.043	0.087					0.348				0.391		
M. domestica modern	M/M	86	0.198			0.023						0.081	0.012			0.686		
M. domestica cider apples	M/M	25	0.400									0.040				0.560		
Total *M. domestica*		422	0.135			0.007	0.005				0.012	0.239	0.007			0.576	0.017	0.002
M. kirghisorum	M/M	3											0.667	0.333				
M. orientalis	M/M	10											0.600	0.300	0.100			
M. prunifolia	M/M	9				0.222			0.333				0.111			0.333		
M. sieversii	M/M	20											0.850	0.100		0.050		
M. baccata	M/B	11	0.182	0.364	0.091	0.182			0.182									
M. hupehensis	M/B	3	0.667									0.333						
M. mandschurica	M/B	3		0.333		0.333			0.333									
M. florentina	S/F	1	1.000															
M. fusca	S/K	2								1.000								
M. kansuenses	S/K	1													1.000			
M. toringoides	S/K	3				0.667		0.333										
M. transitoria	S/K	2				1.000												
Hybrids *M.sylvestris–M. domestica*	—	71	0.464			0.007					0.013	0.170	0.007			0.340		
Feral *M. domestica*	—	21	0.190			0.048						0.190				0.571		

Sec/Ser, section and series according to Phipps *et al*. (1990). M, *Malus*; B, Baccata; S, Sorbomalus; F, Florentinae; K, Kansuenses.

summarizes the frequency of chloroplast haplotypes across species and categories of origin. Eleven haplotypes were detected in series *Malus* only, two haplotypes (cpht2 and cpht3) in series Baccata only and one haplotype (chpt6) in series Kansuenses only. Although this indicates that some haplotypes can be useful to differentiate the different series, these results should be taken with care given the limited number of accessions from series Baccata, Florentinae and Kansuenses that were included in the study.

M. sylvestris and *M. domestica* share eight haplotypes (cpht1, cpht5, cpht9, cpht10, cpht11, cpht14, cpht15, cpht16), although with different frequencies. However, the same three chloroplast haplotypes (cpht1, cpht10 and cpht14) accounted for more than 95% of the accessions of each species. These haplotypes also occur in other *Malus* species but only in one of the analysed *M. sieversii* accessions (cpht14). Two haplotypes (cpht4 and cpht8) are restricted to either *M. sylvestris* or *M. domestica*. Cpht4 is absent from *M. sylvestris* but is shared by *M. domestica*, *M. prunifolia*, *M. baccata*, *M. mandschurica*, *M. toringoides* and *M. transitoria*. Cpht8, which is absent in *M. domestica*, is shared by *M. sylvestris* and *M. fusca*. Cpht11, which is the most frequent haplotype of *M. sieversii*, was also present in three *M. domestica* and in four *M. sylvestris* genotypes.

Almost 60% of the trees sampled in nature but identified as feral *M. domestica* cultivars in the STRUCTURE analysis of nuclear microsatellites carried cpht14 (last row of Table 4), which is also the most frequently found in the cultivar sample. Trees identified as hybrids *M. sylvestris–M. domestica* carried in most cases either cpht1 (frequently found in *M. sylvestris*) or cpht14.

The distribution of haplotypes 5, 9, 15 and 16, which were only detected in *M. domestica* and *M. sylvestris*, is remarkable. Cpht5 was detected three times: in one Danish *M. sylvestris* individual and in two Danish cultivars. Cpht16 was detected only in one Belgian *M. sylvestris* tree and one Belgian cultivar. Cpht9 was detected in five Belgian cultivars and four Belgian *M. sylvestris* individuals. Two Belgian hybrids between *M. sylvestris* and *M. domestica* also carried cpht9. Cpht15 was detected only in seven old Belgian cultivars and in one Belgian *M. sylvestris* accession. Of these four chloroplast haplotypes, only cpht9 was detected in hybrids between *M. sylvestris* and *M. domestica*. These results suggest a close relationship between the two species, even at the local level.

Finally, *M. sylvestris* accessions belonging to the three major chloroplast haplotypes (cpht 1, 10 and 14) were significantly different for nDNA introgression level ($F = 11.80$, $P < 0.001$). Trees carrying cpht1 had significantly lower percentage of their genotypic information attributed to the cultivated gene pool than trees carrying cpht10 or cpht14 (Table 5). This value (0.087) was also lower than the average percentage of assignment to the cultivated gene pool found in *M. sylvestris* (0.13, Table 4). The same analysis was done for *M. domestica* but for this species the groups carrying different chloroplast haplotypes were not significantly different ($F = 0.001$, $P = 0.999$) with regards to the percentage of assignment to the cultivated sample.

Table 5 Comparison of introgression at the nuclear level for *Malus sylvestris* and *Malus domestica* accessions belonging to the three major chloroplast haplotypes

Cpht	Mean percentage assigned to *M. domestica* gene pool*	Standard error
M. sylvestris		
1	0.087[a]	0.008
10	0.190[b]	0.032
14	0.206[b]	0.022
M. domestica		
1	0.950[c]	0.010
10	0.949[c]	0.113
14	0.949[c]	0.005

*Based on nSSR and STRUCTURE results; a, b, c: different letters indicate significantly different values ($P < 0.05$).

Levels of chloroplast diversity

The overall gene diversity was significantly higher ($P < 0.05$) for *M. baccata* and *M. prunifolia* than for *M. domestica* ($h = 0.59$), *M. sylvestris* ($h = 0.52$) and *M. orientalis* ($h = 0.60$). The gene diversity found in *M. sieversii* ($h = 0.28$) was significantly lower ($P < 0.05$) than in any other species analysed (Table 6). No significant differences were found between *M. domestica* and *M. sylvestris*. However, among *M. sylvestris* origins, the two Belgian origins contained significantly higher levels of gene diversity than other origins. For these two origins, the levels of gene diversity were similar to the overall value found for *M. domestica*.

Discussion

Overall levels of cpDNA polymorphism

All origins considered in this study, with the exception of the *M. sylvestris* sample from Germany, displayed some degree of chloroplast diversity. The gene diversity results illustrate the high levels of polymorphism present in the genus *Malus* in the chloroplast regions analysed. However, the actual figures might be much higher, as we did not perform sequence analysis. Our choice was to develop simple PCR and restriction-based tests which enabled us to analyse specific polymorphisms in numerous samples without the need to generate sequence information. By doing this, we have unravelled polymorphisms that might have remained undetected if sequence information had been generated for only a limited number of individuals of each species. On the other hand, sequence information is

Table 6 Chloroplast diversity in *Malus* species and samples. FL, Flanders (north of Belgium); WL, Wallony (south of Belgium)

Category of origin	*N*	*h*	SE(*h*)	*r*(8)
M. sylvestris Belgium (WL)	225	0.570[1]	0.029	2.86
M. sylvestris Belgium (FL)	207	0.556[1]	0.022	2.53
M. sylvestris Denmark	73	0.158[2]	0.057	1.63
M. sylvestris France	19	0.281[2]	0.116	1.83
M. sylvestris Germany	9	0.000	0.000	0.00
M. sylvestris Central Asia	9	0.389[2]	0.164	2.00
Overall *M. sylvestris*		0.515[a]		2.61
M. domestica Belgium (NBS)	139	0.579	0.026	2.63
M. domestica Belgium (CRA)	149	0.604	0.034	3.09
M. domestica Denmark	23	0.731	0.057	3.66
M. domestica Modern	86	0.489	0.055	2.62
M. domestica Cider apples	25	0.547	0.054	2.31
Overall *M. domestica*		0.594[a]		2.97
M. orientalis	10	0.600[a]	0.131	2.80
M. prunifolia	9	0.806[b]	0.089	3.89
M. sieversii	20	0.279[c]	0.123	2.05
M. baccata	11	0.836[b]	0.080	4.56

All parameters were calculated with CONTRIB (Petit *et al*. 1998). *h*: gene diversity, se(*h*): standard error of *h*; *r*(8): allelic richness after rarefaction to a common sample size of 8 genes. a, b, c and 1, 2: different letters and numbers indicate significantly different levels of diversity. Letters refer to differences between species. Numbers refer to differences among categories of origin within each species.

still required to determine the levels of homoplasy present, especially in the restriction patterns of the *trn*H/*trn*K1 and *trn*D/*trn*T regions.

Relationship between Malus sylvestris *and* Malus domestica

The microsatellite analysis of this large set of apple genotypes and subsequent Bayesian assignment approach confirmed our former results (Coart *et al*. 2003) based on a small set of *Malus* trees. Hybridization between *M. sylvestris* and *M. domestica* is present [detected in 11% (71 of 634) of genotypes sampled as *M. sylvestris*] but both species currently represent distinct gene pools across Europe. However, chloroplast information suggests a much closer relationship between *M. sylvestris* and its cultivated relative *M. domestica* than up to now appreciated. Both species share eight chloroplast haplotypes, three of which are frequently found in both species. In addition, local sharing of rare haplotypes was established in this study.

The (local) sharing of chloroplast haplotypes in congeneric species despite limited evidence of introgression at the nuclear level has often been reported for tree species, the most thoroughly studied being the case of the white oaks *Quercus robur* and *Quercus petraea* (Petit *et al*. 2003). The current pattern of chloroplast variation in *M. sylvestris* could therefore be the imprint of ancient hybridization with the omnipresent domesticated apple, especially in Belgium. This would explain the significantly higher levels of chloroplast diversity of Belgian *M. sylvestris* samples compared to other *M. sylvestris* origins (mean $h = 0.59$). Ancient hybridization is also a likely explanation for the significantly higher percentage of introgression found in *M. sylvestris* trees that carry cpht10 and cpht14 (i.e. the most abundant haplotypes among *M. domestica* genotypes) than *M. sylvestris* trees carrying cpht1 (the most frequent haplotype in *M. sylvestris*).

On the contrary, in many other crop–wild relative complexes studied, lower cpDNA diversity was observed in cultivars than in their wild relatives (e.g. in olive, Amane *et al*. 1999; in soybean: Xu *et al*. 2001). Similarly, in a study of cpDNA variation in domesticated cherry and wild *Prunus avium* populations, three haplotypes were detected among cherry cultivars (Panda *et al*. 2003). These haplotypes were also the haplotypes most frequently found in wild cherries, from which cultivars have been derived. However, as expected, the wild species showed higher cpDNA diversity, with mutational combinations resulting in a total of 16 different haplotypes (Mohanty *et al*. 2001; Panda *et al*. 2003).

An explanation for the extensive sharing of chloroplast haplotypes found in the present study is the use of local *M. sylvestris* genotypes in (local) apple breeding. The fact that old cultivars tend to comprise more cpDNA diversity than modern cultivars ($h = 0.58, 0.60, 0.73$ for different samples of old cultivars, and $h = 0.49$ for modern cultivars) could also be the result of the former use of local trees in apple breeding, whereas modern breeding programmes focus on a limited domesticated gene pool. For grape cultivars, multiple origins of domestication were also suggested from the detection of local wild genetic background in local cultivars (Sefc *et al*. 2000). The high cpDNA diversity detected in *Malus* cultivars could be explained by the hypothesis of the complex hybrid origin of *M. domestica* (briefly reviewed in Robinson *et al*. 2001 and Hokanson *et al*. 2001), and thus by admixture of genetic information from different species.

In conclusion, the high levels of haplotype sharing between *M. sylvestris* and *M. domestica* indicate interspecific gene flow, which is probably bidirectional and brought about by the use of (local) wild *Malus* genotypes for the (local) cultivation process of apple and the later cytoplasmic introgression of chloroplast haplotypes into *M. sylvestris* populations from the domesticated apple.

Relationship between M. sieversii *and* M. domestica

The three most frequent chloroplast haplotypes of *M. domestica* and *M. sylvestris* were nearly absent in the analysed *Malus sieversii* accessions. This was unexpected, because this species had previously been considered to represent the most important maternal progenitor of the domesticated

apple, mainly based on DNA polymorphisms in the *mat*K region (Robinson *et al*. 2001; Forte *et al*. 2002; Harris *et al*. 2002). Furthermore, our analyses of 10 nuclear microsatellite loci for the same genotypes demonstrated that the nuclear genome of *M. sieversii* is almost as divergent from that of *M. domestica* as the genome of *M. sylvestris*. In conclusion, our results do not support the former hypothesis and hence reopens the exciting discussion on the origin of apple cultivars.

Future prospects

To further unravel the phylogenetic relationship between these apple species and the extent of hybridization between them, the establishment of the nature of chloroplast polymorphisms through sequencing (as in the case of the *trn*T/*trn*D region) would provide useful information. This would permit an in-depth study of the relationship between different chloroplast haplotypes. It has indeed been shown that estimates of genetic relationships in which the similarities between haplotypes are not taken into account result in the loss of useful information, especially at broad geographic scales (Petit *et al*. 2005). Also promising is the analysis of allelic associations at linked nuclear markers. This can potentially provide further insight into the amount and regional distribution of recent hybridization and introgression events since one would expect significant associations between linked markers under a scenario of (ongoing) introgression but not from common descent caused by ancient use of the wild species in the domestication process (Rieseberg *et al*. 2000; Falush *et al*. 2003). Finally, the analysis of a broader sample of *M. sieversii* will provide clues about the relationship between this species and *M. domestica*.

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Supplementary material

The supplementary material is available form http://www.blackwellpublishing.com/products/journals/suppmat/MEC/MEC2924/MEC2924sm.htm

Table S1 Wild *Malus* accessions obtained from USDA-ARS at Cornell University, Geneva, USA

Table S2 Primers used for amplification and sequencing of the *mat*K region

Figure S1 Detection of the presence/absence of duplication II in the *mat*K region.

Figure S2 PCR-RFLP restriction patterns obtained with HK1/*Eco*RI and DT/*Mse*I.

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