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Comparative Molecular Genetic Analysis of Apple Genotypes Maintained in Germplasm Collections

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Abstract Apple (*Malus* × *domestica*) is economically one of the most important fruit crops in the world, with several thousand described varieties of which only a small number is commercially grown. Characterisation of genetic resources of apple is usually based on morphological traits, which are modulated by environmental and ecological factors, and for this reason pomological description needs to be complemented by molecular approaches. Ten accessions assigned to the apple cultivars 'Antonovka', 'Laxton's Superb' and 'Worcester Pearmain' were derived from several germplasm collections in Europe and analysed at 14 variable microsatellite loci. In order to verify their assignment, the molecular genetic data were compared to a database containing molecular genetic profiles of reference varieties. Within the five accessions of different origin maintained as 'Antonovka', two genotypes were identified, which could be assigned as the common 'Antonovka' and 'Antonovka polutorafuntowaja'. All the three accessions of 'Laxton's Superb' displayed the same genotype that was consequently considered to be authentic, and the comparison with the entries of the database enabled to reveal the probable parent pair for this cultivar. For the two accessions of 'Worcester Pearmain', the comparative database approach allowed to recognise a misidentification in one of the two germplasm collections. A comparative analysis of different accessions of a cultivar from independent origins and the constitution of a database are required, in order to contribute to a reliable determination of apple cultivars maintained in germplasm collections.

Keywords *Malus* × *domestica* · Cultivar identification · Molecular Markers · Microsatellite DNA · Breeding · 'Antonovka' · 'Laxton's Superb' · 'Worcester Pearmain'

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Vergleichende molekulargenetische Analyse von Apfel-Genotypen aus Sortensammlungen

Zusammenfassung Der Apfel (*Malus* × *domestica*) ist weltweit eine der wichtigsten Obstarten, von der mehrere tausend Sorten beschrieben sind, aber nur wenige davon im Erwerbsobstbau angebaut werden. Die Charakterisierung dieser genetischen Ressourcen beruht meist auf morphologischen Merkmalen, welche durch Umwelteinflüsse und ökologische Faktoren moduliert werden. Die pomologische Beschreibung muss deshalb durch molekulargenetische Analysen ergänzt werden. Zehn Akzessionen der mutmaßlichen Apfel-Sorten 'Antonowka', 'Laxton's Superb' und 'Worcester Parmäne' aus verschiedenen europäischen Genbanken wurden an 14 Mikrosatelliten-Loci untersucht. Um diese Apfel-Akzessionen identifizieren zu können, wurden deren molekulargenetische Daten mit den molekulargenetischen Profilen einer Datenbank von Referenzsorten verglichen. Die fünf als 'Antonowka' bezeichneten Akzessionen unterschiedlicher Herkunft konnten zwei Genotypen, der gewöhnlichen 'Antonowka' und der 'Antonowka polutorafuntowaja', zugeordnet werden. Alle drei Akzessionen der Sorte 'Laxton's Superb' zeigten denselben Genotyp,

der deshalb als authentisch angenommen wurde, und eine Analyse mit Hilfe der Referenz-Datenbank ermöglichte die Zuordnung der wahrscheinlichen Elternsorten. Für die Sorte ‚'Worcester Parmäne' konnte, anhand der genetischen Profile und des Vergleiches mit der Referenz-Datenbank, eine der beiden Genbank-Akzessionen als Fehlbestimmung aufgedeckt werden. Vergleichende molekularbiologische Analysen verschiedener Akzessionen und aus verschiedenen Genbanken, sowie die Errichtung einer Referenz-Datenbank sind notwendig, um eine verlässliche Bestimmung der Apfelsorten in Sortensammlungen durchführen zu können.

Schlüsselwörter Apfelsorten · *Malus* × *domestica* · Sortenbestimmung · Molekulare Marker · Züchtung · 'Antonovka' · 'Laxton's Superb' · 'Worcester Parmäne'

Introduction

Apple (*Malus* × *domestica* Borkh) is economically one of the most important fruit trees of temperate climate zones with an extensive cultivation history (Janick et al. 1996; Dalla Via and Baric 2012). Although apple growing areas are widespread throughout the world, global production is dominated by only a small number of cultivars such as 'Golden Delicious', 'Granny Smith' or 'Red Delicious' (O'Rourke 2003), and as a result the gene pool of local cultivars has been considerably reduced (Hokanson et al. 1998, 2001). For this reason, there have been efforts in different apple growing regions to collect the remaining local genetic diversity of this fruit crop (e.g. Baric et al. 2008; Guarino et al. 2006; Guilford et al. 1997; Pereira-Lorenzo et al. 2007) and to preserve this diversity in germplasm collections. The role of collections is, however, not only to maintain the agricultural biodiversity as cultural heritage for the future, but also to accurately describe and characterise the accessions in order to use them as potential donors of desirable traits (such as disease resistance or fruit quality) in breeding programmes (Hokanson et al. 2001; Kumar et al. 2012).

Characterisation of apple cultivars is generally based on morphological traits, which can be modulated by environmental and ecologsical factors, so that pomological descriptions need to be complemented by the application of molecular approaches (Baric et al. 2009, 2012; Guilford et al. 1997). One of the molecular tools currently widely employed are microsatellite DNA markers or SSRs (Short Sequence Repeats), which are highly robust and reproducible (Baric et al. 2008, 2011; Guarino et al. 2006; Guilford et al. 1997; Hokanson et al. 1998; Pereira-Lorenzo et al. 2007; Wünsch and Hormaza 2002) and are not influenced by environmental factors. Apart from the determination of genetic identity of single accessions, microsatellites are suitable for the assessment of genetic relationships or estimates of genetic diversity within a germplasm collection (Hokanson et al. 2001). In order to deploy molecular markers for the determination of unidentified or misidentified apple trees, first a database with molecular genetic profiles of well-determined reference cultivars from independent germplasm collections needs to be established (Baric et al. 2009). The second step is to carry out comparisons of genetic profiles of unknown or doubtful samples with the confirmed entries of the database, which allows reliable determination.

In the present study, accessions assigned as cultivars 'Antonovka', 'Laxton's Superb' and 'Worcester Pearmain' were obtained from several germplasm collections in Europe and analysed at 14 variable microsatellite loci. The aim was to illustrate the usefulness of the application of molecular tools in combination with a database containing molecular genetic profiles of reference cultivars, for the identification and characterisation of apple cultivars as well as for authenticity assessment.

Material and Methods

This study concentrated on ten apple accessions assigned to the cultivars 'Antonovka', 'Laxton's Superb' and 'Worcester Pearmain'. Four accessions of 'Antonovka' were obtained from the germplasm collections of the Institute of Horticulture and Viticulture, University of Natural Resources and Applied Life Sciences in Vienna—Austria (BOKU), the National Fruit Collection in Brogdale—United Kingdom (BC), the Kompetenzzentrum Obstbau-Bodensee in Bavendorf—Germany (KOB), and the Obst-Kulturweg in Rosenheim—Germany (OKR). In addition, a sample was taken from a field-grown 'Antonovka' tree in Val di Non (Trentino, northern Italy) (VN). Three accessions of 'Laxton's Superb' were obtained from BC, KOB and OKR, and two accessions of 'Worcester Pearmain' from BC and Bundesamt für Wein- und Obstbau Klosterneuburg—Austria (KN).

Genomic DNA was isolated from fresh leaves using "NucleoSpin Plant Mini Kit" following the manufacturer's protocol (Macherey-Nagel, Düren, Germany) and analysed at 14 microsatellite loci (CH01c06, CH01d08, CH01f02, CH01f07a, CH02b10, CH02c02a, CH02c09, CH02c11, CH02d08, CH02d12, CH02h11a, CH03a04, COL and CH01h01) (Liebhard et al. 2002). Separation of fragments was performed on a capillary electrophoresis instrument CEQ 8000 (Beckman Coulter, Fullerton, CA, USA) and allele sizes were assigned with the Fragment Analysis Software Version 8.0. Finally, the molecular genetic profiles of the samples were first compared to each other and then to the profiles contained in the database of the Research Centre

for Agriculture and Forestry Laimburg using a cross-tabulation matrix created in Microsoft Access (Baric et al. 2009).

Results and Discussion

The analysis of ten apple accessions assigned to the cultivars 'Antonovka', 'Laxton's Superb' and 'Worcester Pearmain' at 14 microsatellite loci revealed five distinct diploid genotypes (Tables 1, 2, and 3).

The five accessions of 'Antonovka' displayed two different genotypes, one found for the accessions from BC and OKR, and the other for the accessions from BOKU, KOB and the field sample VN (Table 1). The identification of different genotypes for 'Antonovka' is in fact not surprising, since this name is associated with a large number of different variants originating from Russia (Rolff 2001; Votteler 1986). Bus et al. (2012) have recently reviewed the 'Antonovka' cultivar complex, which has been widely used as a source of scab resistance by apple breeders all around the world. Six of the microsatellite loci analysed by Bus et al. (2012) and herein were identical (see Table 1). The comparison of molecular genetic profiles suggests that the 'Antonovka' accessions from BC and OKR might represent the common 'Antonovka' APF22, while those from BOKU, KOB and VN might be identified as 'Antonovka polutorafuntowaja', which is characterised by a larger fruit size. At three loci, the genetic profiles of 'Antonovka' BC and OKR were identical with 'Antonovka' APF22, while those of 'Antonovka' BOKU, KOB and VN were identical with 'Antonovka polutorafuntowaja' (Table 1). At locus CH02c09, the allele sizes differed consistently by a single base pair (+1 bp in the present study). This was also valid for

Table 1 Comparison of two different genotypes of 'Antonovka' found in accessions from germplasm collections BOKU, KOB, BC and OKR, and the field-grown tree VN. Identical alleles found in both genotypes are represented in italics. In the last two columns, data for six microsatellite loci that were obtained by Bus et al. (2012) are given for the accessions Antonovka APF22 and Antonovka polutorafuntovaya. Alleles are listed only once if a cultivar was homozygous at a particular locus

SSR Locus	'Antonovka' (BOKU, KOB, VN)	'Antonovka' (BC, OKR)	Antonovka APF22[a]	Antonovka polutorafuntovaya[a]
CH01c06	154:*162*	156:*162*	n.a.	n.a.
CH01d08	*240:254*	*240:254*	n.a.	n.a.
CH01f02	*180*:188	170:*180*	170:*180*	*180*:188
CH01f07a	*189:193*	*189:193*	n.a.	n.a.
CH02b10	113:*131*	*131*:155	*132*:159	114:*132*
CH02c02a	*179*:193	*179*:183	n.a.	n.a.
CH02c09	*247*:257	*247*:251	*246*:250	*246*:256
CH02c11	*218*:228	*218*:226	*218*:226	*218*:228
CH02d08	220:*250*	*250*	*248*	219:*248*
CH02d12	*199*	*199*	n.a.	n.a.
CH02h11a	*100*:116	*100*:114	n.a.	n.a.
CH03a04	*98*:100	*98*:112	n.a.	n.a.
COL	*229*	*229*	n.a.	n.a.
CH01h01	*118*	*118*	*118*	*118*

[a]Data from Bus et al. (2012)

n.a. not assessed

Table 2 Comparison of the molecular genetic profile of the cultivar 'Laxton's Superb' with its potential parent cultivars. Identical alleles found in the progeny (Laxton's Superb) and each of the two parental cultivars are represented in italics. Alleles are listed only once if a cultivar was homozygous at a particular locus

SSR Locus	'Laxton's Superb' (BC, KOB, OKR)	'Cox's Orange Pippin' (BC, DP, KOB)	'Cellini' (AGES, DP, KN)
CH01c06	*160:162*	*160*:170	154:*162*
CH01d08	*254:272*	240:*254*	254:*272*
CH01f02	*180:208*	206:*208*	*180*:190
CH01f07a	*173:177*	*173*:195	*177*:179
CH02b10	*119:143*	*119*:131	123:*143*
CH02c02a	*155:165*	*155*	*165*:183
CH02c09	*235:259*	*235*:259	241:*259*
CH02c11	*220:226*	*220*	220:*226*
CH02d08	*220:258*	*220*	220:*258*
CH02d12	*177:199*	*177*	177:*199*
CH02h11a	*98:116*	*98*:122	114:*116*
CH03a04	*98:104*	*104*:120	*98*:120
COL	*219:229*	*229*	*219*:221
CH01h01	*134*	122:*134*	118:*134*

Table 3 Comparison of genotypes found for two accessions of the cultivar 'Worcester Pearmain' with two of its progeny cultivars. Alleles of 'Worcester Pearmain' BC found in 'Discovery' and 'Lord Lambourne' are indicated with "A" and "a", and alleles of 'Worcester Pearmain' KN found in 'Discovery' and 'Lord Lambourne' are indicated with "B" and "b", respectively

SSR Locus	'Worcester Pearmain' (BC)	'Worcester Pearmain' (KN)	'Discovery' (BC, KN)	'Lord Lambourne' (BC, BOKU, DP)
CH01c06	156^{a}:172^{A}	160^{B}:162^{b}	160^{B}:172^{A}	156^{a}:162^{b}
CH01d08	272:294^{Aa}	240^{Bb}:260	240^{B}:294^{A}	240^{b}:294^{a}
CH01f02	188:208^{Aa}	206	184:208^{A}	208^{a}
CH01f07a	193^{Aa}	179:195^{B}	193^{A}:195^{B}	193^{a}
CH02b10	129^{Aa}:137	119:137	129^{A}	129^{a}:131
CH02c02a	141^{a}:179^{A}	155^{b}:179^{B}	177:179^{AB}	141^{a}:155^{b}
CH02c09	235^{a}:247^{Aa}	235^{b}:259^{B}	247^{A}:259^{B}	235^{ab}:247^{a}
CH02c11	226^{A}:230^{Aa}	220:232	226^{A}:230^{A}	230^{a}:240
CH02d08	214:254^{Aa}	258^{b}	232:254^{A}	254^{a}:258^{b}
CH02d12	191:199^{Aa}	199^{Bb}:201	199^{AB}	199^{ab}
CH02h11a	98^{a}:118^{A}	98^{b}:122^{B}	118^{A}:122^{B}	98^{ab}
CH03a04	98^{A}:112^{a}	104:112^{b}	98^{A}:110	112^{ab}:120
COL	229^{Aa}	221:229^{Bb}	229^{AB}:239	229^{ab}
CH01h01	116^{a}:134^{A}	126:134^{B}	120:134^{AB}	116^{a}:124

three alleles of locus CH02b10 (−1 bp in the present study), while the larger allele of 'Antonovka' APF22 and 'Antonovka' BC and OKR differed by 4 bp. At locus CH02d08, 1-bp difference was found for the shorter allele and 2-bp difference for the longer allele. The incongruences at three loci can be explained by the different capillary electrophoresis systems employed in the two studies (Baric et al. 2008) and by the occurrence of 'allelic drift' which can impede the accuracy of allele binning in automated electrophoresis systems (Idury and Cardon 1997). The latter seems to be the case for loci CH02b10 and CH02d08, where Bus et al. (2012) have reported genotypes composed of both even and uneven fragment lengths for dinucleotide-repeat microsatellite markers (Table 1).

It is assumed that 'Antonovka polutorafuntowaja' is a direct progeny of the common 'Antonovka' (Bus et al. 2012). The fact that the two 'Antonovka' genotypes obtained in the present study share a common set of alleles at all 14 microsatellite loci (Table 1) further suggests that the identification of the accessions analysed is correct. Moreover, photographs of fruit are available for the accessions OKR and VN (not shown), and their comparison with the descriptions of common 'Antonovka' and 'Antonovka polutorafuntowaja' by Votteler (1986) offers additional evidence that the two genotypes were accurately identified.

The comparison of the genetic profiles from the three accessions of 'Laxton's Superb' deriving from BC, KOB and OKR resulted in a single genotype that was consequently considered to be authentic. This Victorian British apple cultivar was bred by Thomas Laxton and is claimed to be a cross of 'Wyken Pippin' × 'Cox's Orange Pippin' (Morgan and Richards 1993). This cultivar was analysed in a recent study in which pedigreed apple cultivars were genotyped at 80 microsatellite markers to assess the authenticity of their identity and parentage (Evans et al. 2011). Parentage discrepancies were suspected for 'Laxton's Superb' but could not be proven due to the insufficient sample number (Evans et al. 2011). Therefore, we compared the genotype of this cultivar at 14 microsatellite loci with the molecular genetic profiles of its potential parent cultivars present in the database of the Research Centre Laimburg (Baric et al. 2009). No data was available for one of the presumed parents 'Wyken Pippin', but the database contained a molecular genetic profile obtained from three accessions of 'Cox's Orange Pippin' collected from the germplasm collections BC, KOB and the Institute of Fruit Breeding Dresden-Pillnitz—Germany (DP) (see Table 2). The comparison of the molecular genetic profiles revealed that 'Laxton's Superb' and 'Cox's Orange Pippin' shared a set of alleles at each of the 14 microsatellite loci, supporting the previous parentage assumption. Subsequently, the molecular genetic profile of the cultivar 'Cellini' was included in the comparison, which is the second parent of 'Laxton's Exquisite', another cultivar arising from Thomas Laxton's breeding programme (Morgan and Richards 1993). The three accessions of the cultivar 'Cellini' were obtained from the germplasm collections DP, KN and the Austrian Agency for Food Safety, AGES Linz—Austria (AGES). The remaining set of alleles of 'Laxton's Superb' matched exactly with an allelic set of 'Cellini' (see Table 2). Our data thus suggests that the cultivar 'Laxton's Superb' is a progeny of 'Cox's Orange Pippin' × 'Cellini' and represents a sister cultivar of 'Laxton's Exquisite'. In order to verify that 'Wyken Pippin', where data was unavailable for analysis, is not the true parent of 'Laxton's Superb', accessions of this cultivar from different germplasm collections will be analysed in the future. In addition, accessions of the cultivar 'Laxton's Exquisite' will be investigated, to determine whether it was derived from the same parents as 'Laxton's Superb'.

The analysis of the two accessions of 'Worcester Pearmain' resulted in the discovery of two distinct genotypes, meaning that the 'Worcester Pearmain' accession from BC showed a different molecular genetic profile compared to the 'Worcester Pearmain' accession from KN (Table 3). Consequently, we aimed to assess which of the two ambiguous genotypes could represent the authentic cultivar. This was carried out by comparing the molecular genetic profiles of the two 'Worcester Pearmain' accessions with the genotypes of two cultivars, 'Discovery' and 'Lord Lambourne', present in the database of the Research Centre Laimburg (Table 3). The 'Discovery' genotype was found in two accessions of this cultivar deriving from the germplasm collections BC and KN, while the 'Lord Lambourne' genotype was found in three accessions collected in the collections BC, BOKU and DP. The cultivars 'Discovery' and 'Lord Lambourne' were selected due to the fact that 'Worcester Pearmain' was frequently used as a parent in breeding programmes and these two cultivars were recently confirmed to represent its progeny (Evans et al. 2011). In the event that one of the two 'Worcester Pearmain' genotypes identified in our study was authentic, we would expect to find a complete haploid set of its alleles in both progeny cultivars. In fact, the 'Worcester Pearmain' accession from BC fulfilled this expectation, while the accession from KN showed a correspondence at only 9 of the 14 loci with both progenies. Thus, we conclude that the accession from BC very likely represents the authentic 'Worcester Pearmain', while the accession from KN was most probably misidentified. These assumptions, however, will be further confirmed by the analysis of additional accessions of the cultivar 'Worcester Pearmain'.

In conclusion, the present study confirms that microsatellite markers are a useful tool for studying the descent, the identity and the correctness of assignment of apple cultivars in germplasm collections. It particularly emphasises the need for a comparative analysis of different accessions of a cultivar from independent collections and for the con-

stitution of a database, in order to contribute to both, efficient management of genetic resources and reliable selection of parental cultivars for breeding programmes.

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