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# Assessment of the genetic diversity of native apple cultivars in the south eastern ranges of the Alps with three selected microsatellite loci

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## Summary

The regional diversity of native apple cultivars in parts of the south eastern ranges of the Alps (Styria, Austria and northern parts of Slovenia) was examined. As the application of conventional pomological methods to characterise cultivars may sometimes be ambiguous, we regard the application of molecular methods to be essential for thorough cultivar diversity assessments. Five hundred samples were collected from different climatic and edaphic regions and analysed using three selected microsatellite loci. With this approach we were able to distinguish 190 named varieties at which we chose 50 as reference varieties. The high diversity of native races suggests that the Southern alpine ranges represent a „hot spot“ of cultivar diversity. This can be attributed to historical effects and the local persistence of a traditional management practice with orchards of widely spaced and old-grown trees of various races. Because these „old“ native races could harbour interesting genetic traits (pathogen resistance, taste, etc.) that will be important in future food production, measures for their conservation are overdue. Our approach will not only show which local cultivars/genotypes require rapid action for their protection, but due to the international nature of our project we can also show which old and untraceable local names in different languages correspond with the same cultivars.

## Introduction

Representing one the oldest and most widely cultivated temperate fruit crops, apple (*Malus* x *domestica* Borkh.) developed into a great diversity of cultivars. Of these, only a few are grown in mass production to fit the requirements of the international market of today. In former times each country or region maintained an own local stock of cultivars (JANICK et al., 1996). From these, ecological types could be selected which were particularly adapted to local environments and requirements. These old varieties and land races have been used by humans since hundreds of years and therefore hold a special position in the cultural landscape heritage of any region where apple has a history of cultivation. However, for complex reasons the genetic diversity of the domesticated apples has decreased dramatically in the last few years (HOKANSON et al., 2001).

The colline to montane elevations of the south eastern Alpine borders are a perfect example for a historically rich diversity of apple cultivars. Originating from Styria (Austria), which might be regarded as “hot-spot” of apple diversity, apple cultivation was extended towards Slovenia, northern Italy and parts of Croatia during the times of the Habsburgian empire. Some of the races were better adapted than others to grow at different altitudes, to withstand high light, to resist massive damage by pathogens, etc. A tremendous number of names of these cultivars are found in the literature (ROLFF, 2001; HARTMANN, 2003; GRILL and KEPPEL, 2005), or can be collected in interviews with local farmers. Since there has never been a comprehensive and consistent collection of cultivars for comparison, the naming practice resulted in many confusion, especially with rarely grown cultivars. E.g. old horticultural names were sometimes replaced by informal local names, which persisted through times, or wrong determinations could result in the dissemination of shoots with wrong names. The taxonomic chaos of informal vernacular names lead to a considerable uncertainty about estimates of cultivar diversity in the south-eastern alpine ranges.

Because native apple cultivars represent a rich stock of exploitable genetic resources, the conservation of old and local cultivars should be seen as an important task to ensure a sustainable environment. It is clear that a general screening of the diversity of old local apple varieties is an indispensable first step before an effective conservation management can be outlined (HODGKIN et al., 2001). However, and especially in the case of apple, the traditional determination methods, including measurements and objective descriptions of fruit and tree characteristics, are time-consuming and not always consistent (KENIS et al., 2001). This is due to the variability in their fruit parameters (e. g. colour, shape, tightness), which is strongly influenced by ecological and physiological parameters, such as soil composition, exposition, climatic fluctuations. To complement the morphological characterisation, isoenzymes and alloenzymes have previously been included (ROYO and ITOIZ, 2004), but their patterns may again depend on environmental factors. The emergence of new PCR-based molecular markers, such as randomly amplified polymorphic DNA (RAPDs), simple sequence repeats (SSRs), and amplified fragment length polymorphisms (AFLPs), has created the opportunity for fine-scale genetic characterizations of germplasm collections that were previously impossible (HOKANSON et al., 1998).

An alternative method for characterisation and identification of old native apple cultivars which is independent from changing environmental influences is provided by the anlaysis of length variation at microsatellite loci. By their polymorphic nature, microsatellites became popular for various genetic approaches (genomic mapping, study of genomic instability in cancer cells, population genetics, forensics and conservation biology; SHINDE et al., 2003). Microsatellites, or simple sequence repeats (SSRs), are short stretches of DNA, consisting of tandemly repeated nucleotide units. The high levels of variation in the number of repeats are thought to arise from replication slippage, i.e. the transient dissociation of the replicating DNA strands followed by misaligned reassociation (ELLEGREN, 2004). A general method for the detection of polymorphic microsatellites is based on PCR amplification using a unique pair of primers flanking the simple sequence repeats (WEBER and MAY, 1989). As they are uniformly distributed, hypervariable, codominant and abundant in most genomes, they are interesting for studies of apple (GIANFRANCESCHI et al., 1998), and can be used to gain deeper insights in the distribution of phenotypic traits (by QTL analysis) or for an assessment of cultivar diversity.

The aim of this study is to examine the genetic diversity of native apple cultivars by means of microsatellite length variations and to clarify some naming problems of apple varieties in the area of investigation.

## Materials and methods

For the estimation of the genetic diversity of native apple cultivars in the area of investigation (Styria and northern parts of Slovenia) a

basic inquiry was carried out. We sent out a standardised questionnaire to all agricultural schools to obtain information about the cultivars composing these special cultural landscape. From these collected data we selected varieties for genetic analysis by microsatellites according to specified criteria, as follows: 1. the age of the trees (80 - 100 years old), 2. unclear local names, 3. special usage of the fruits (such as dried fruit), 4. occurrence at relatively high altitudes (> 1300 m) or 5. such varieties, which are not yet cultivated in a living bank of genetic resources (arboretum). Varieties determined by pomological experts (S. Bernkopf, Linz and H. Keppel, Graz) were analysed as references for comparison.

From the selected trees for microsatellite analyses we collected young leaves for DNA extraction. The leaves were frozen in liquid nitrogen immediately after harvesting. The lyophilised leaves (Hetosicc Freeze Dryer Type CD 4, Heto Lab Equipment, Birkerod, Denmark) were ground to powder by the Micro-Dismembrator II (B. Braun Biotech International GmbH, Meisungen, Germany) and stored in humidity proof plastic vials at -25°C until DNA extraction. The DNA was isolated from 17 mg of leaf powder using the DNeasy® Plant Mini Kit (Qiagen, Vienna).

For the genetic characterisation of the native apple cultivars by simple sequence repeats we used three different polymorphic primer combinations with high expected heterozygosity (forward and reverse primer), which represent different linkage groups (GIANFRANCESCHI et al., 1998): CH01F02, CH02C06 and CH02D12. The forward primer of each primer pair was labelled at the 5´-end with a fluorescent dye (FAM, HEX or NED). The amplification of the three selected microsatellite loci by PCR followed the conditions published by GIANFRANCESCHI et al. (1998). The specific amplified DNA fragments (microsatellites) were run on a ABI PRISM® 310 Genetic Analyser. The detected fragment lengths were calibrated by a fluorescent labelled size standard (Genescan 500 ROX) which was added to each sample. Sizing of fragments was done with GeneScan®Analysis Software Version 3.1. Because lengths from each microsatellite locus was uniform within an cultivar, differences in the allelic compositions were used for characterisation and identification of old native apple cultivars.

## Results and discussion

Based on the primary inquiry of the native apple cultivars by standardised questionnaires and after selection of relevant varieties (see above) we examined 500 trees by microsatellite analysis. The examined apple cultivars are characterised by their allelic composition at the three selected SSR loci. From the 500 DNA-extractions, which were sampled evenly across the area of investigation, we could differentiate 190 varieties. The application of only three selected microsatellite loci can be regarded as a cost efficient method for a screening of regional apple cultivar diversity.

To characterise unknown cultivars and to clarify the complex synonymy, it is necessary to compare the allelic length of reference samples of varieties with that of cultivars with dubious names. The determination of such references is a difficult task. The selection of samples, which are to become reference samples in the future was carried out in accordance of microsatellite data with morphological fruit specifications (e. g. colour, shape, tightness), and expert knowledge for local varieties (S. Bernkopf, Linz and H. Keppel, Graz). Of the 190 differentiated samples we selected 50 as reference varieties (Tab. 1). This reference data base will allow a secure identification of apples by their allelic composition.

The allelic composition of the cultivars interpreted according to LIEBHARD et al. (2002) as follows. In cases where only one fragment is visible, the allel can be homozygous or an null allel is involved (eg. Ananasrenette at locus CH01F02). In the latter case the individual

**Tab. 1:** Allelic composition of 50 reference apple cultivars (nomenclature according to GRILL and KEPPEL, 2005)

| Cultivar | SSR name | | |
|---|---|---|---|
| | CH01F02 | CH02C06 | CH02D12 |
| Ananasrenette | 183 | 241:249 | 190:198 |
| Baumanns Renette | 169:179 | 215:227 | 182:198 |
| Berner Rosenapfel | 181 | 233:249 | 198:206 |
| Boikenapfel | 179 | 215:233 | 198:200 |
| Champagner Renette | 183 | 249 | 190:200 |
| Charlamowsky | 171 | 205:251 | 176:198 |
| Coulons Renette | 181:183 | 227:237 | 180:210 |
| Damasonrenette | 179:183 | 233:243:253 | 182:198 |
| Danziger Kantapfel | 169 | 229:251 | 180:198 |
| Elise Rathke | 169:183 | 249 | 180:190 |
| Geflammter Kardinal | 169:179 | 215:227:247 | 182:198:210 |
| Geheimrat Dr. Oldenburg | 189 | 249 | 176:190 |
| Gehrers Rambur | 169 | 215:227:249 | 198 |
| Gelber Bellefleur | 173:179 | 233:249 | 194:198 |
| Goldrenette von Blenheim | 183 | 241:249:257 | 198 |
| Grahams Jubiläumsapfel | 173 | 249 | 176:198 |
| Graue Herbstrenette | 179:183 | 233:243 | 182:198:200 |
| Gravensteiner | 181:183 | 215:249 | 198:212 |
| Großer Rheinischer Bohnapfel | 179:183 | 215:249 | 194:198 |
| Grüner Stettiner | 169 | 215:233:255 | 180:194:198 |
| Hagedorn | 179:183 | 215:251 | 176:198 |
| Harberts Renette | 183:185 | 241:249 | 180:198 |
| Haslinger | 169:179:183 | 249 | 176:182:190 |
| Ilzer Rosenapfel | 169 | 259 | 182:198 |
| Jakob Lebel | 179:187:195 | 227:237 | 176:182:198 |
| Jonathan | 169:179 | 233:249 | 190:198 |
| Kalterer Böhmer | 169 | 229:231 | 182:206 |
| Kanada Renette | 179 | 227:247 | 182:190:198 |
| Kardinal Graf Galen | 169 | 249 | 176:206 |
| Karmeliter Renette | 179:183 | 257 | 198 |
| Klöcher Maschanzker | 169:181 | 243:249 | 190:198:206 |
| Kronprinz Rudolf | 169:183 | 241:249 | 176:198 |
| Landsberger Renette | 183:189 | 237:251 | 176:190 |
| Lavanttaler Bananenapfel | 183 | 249 | 206:210 |
| London Pepping | 179 | 215 | 176:206 |
| Odenwälder | 169:193 | 249 | 176:198 |
| Rheinischer Krummstiel | 169:181 | 237:249 | 176:198 |
| Rote Schafnase | 169 | 229:231 | 198:204 |
| Roter Herbstkalvill | 173 | 249 | 182:198 |
| Roter Trierer Weinapfel | 169 | 247 | 180:182 |
| Sauergrauech | 181:183 | 233:247 | 198 |
| Schmidberger Renette | 169:183 | 249 | 198:210 |
| Schöner von Boskoop | 183 | 227:237 | 180:194 |
| Signe Tillisch | 189 | 215:251 | 176 |
| Steirischer Maschanzker | 169:181 | 249 | 198:206 |
| Steirischer Passamaner | 169:171 | 215:231 | 184:198 |
| Wagener Apfel | 169:181 | 249 | 180:206 |
| Weißer Klarapfel | 181:187 | 215:249 | 178:198 |
| Weißer Winterkalvill | 181 | 249 | 182:198 |
| Wintergoldparmäne | 183 | 237:249 | 198:210 |

could be heterozygous and the other allel is not detectable because of mutations in the priming site and prevention of annealing. Two allels represent an diploid heterozygous individual (or a triploid with null allel). Three allels at the same locus of one individual could indicate a triploid heterozygous cultivar or for multilocus SSR (e. g. Damasonrenette at locus CH02C06).

Our data confirm a high diversity of extant apple cultivars in Styria and parts of Slovenia. We could also resolve several nomenclatural problems with our data (Tab. 2). For example the cultivar „Baumanns Renette“ displays the same allelic composition as the collections „Kranzler“, „Türken rot“, „Nikolausapfel“ and „Baumanova reneta“, which represent vernacular names for the regular cultivar term “Baumanns Renette”. A posteriori inspection of pomological characters confirmed the molecular approaches. Any slight differences between the phenotypical descriptions seem to be within the range of modifications of each race.

**Tab. 2:** Examples for discovered synonyms (regular cultivar names are given in bold letters, GRILL and KEPPEL, 2005)

| Cultivar | SSR name | | |
|---|---|---|---|
| | CH01F02 | CH02C06 | CH02D12 |
| **Baumanns Renette** | 169:179 | 215:227 | 182:198 |
| Kranzler | 169:179 | 215:227 | 182:198 |
| Türken rot | 169:179 | 215:227 | 182:198 |
| Nikolausapfel | 169:179 | 215:227 | 182:198 |
| Baumanova reneta | 169:179 | 215:227 | 182:198 |
| **Haslinger** | 169:179:183 | 249 | 176:182:190 |
| Steirischer Pogatschenapfel | 169:179:183 | 249 | 176:182:190 |
| Roter Pogatschenapfel | 169:179:183 | 249 | 176:182:190 |
| Haselapfel | 169:179:183 | 249 | 176:182:190 |
| Breitschädel | 169:179:183 | 249 | 176:182:190 |
| Pogačarica | 169:179:183 | 249 | 176:182:190 |
| **Roter Herbstkalvill** | 173 | 249 | 182:198 |
| Himbeerapfel | 173 | 249 | 182:198 |
| Erdbeerapfel | 173 | 249 | 182:198 |
| Klachlapfel | 173 | 249 | 182:198 |
| Roter Paradiesapfel | 173 | 249 | 182:198 |
| Herzapfel | 173 | 249 | 182:198 |
| Klingler | 173 | 249 | 182:198 |
| Tschepperer | 173 | 249 | 182:198 |
| Rdeči jesenski kalvil | 173 | 249 | 182:198 |
| **Weißer Klarapfel** | 181:187 | 215:249 | 178:198 |
| Butterapfel | 181:187 | 215:249 | 178:198 |

In parallel to SSR-typing of characterised apple varieties their cultivation in an arboretum as a living bank of genetic resources is planned. This will facilitate the conservation and dissemination of genetically valuable native apple cultivars. Another anticipated endeavour is a transregional alignment of the obtained microsatellite data with other research institutes.

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