Identification of Citruses from Montenegro Based on Microsatellite Clustering Analyses

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Abstract
A set of 32 citrus genotypes were selected from Montenegro collection and analyzed by the use of 11 microsatellite markers. Analyses harvested 72 different alleles having 6.5 alleles per locus and discovered presence of 23 unique genotypes. Distance analysis and model-based clustering revealed both the presence of 4 groups: satsuma (*Citrus unshiu* Marc.) mandarins, clementine (*Citrus reticulata*) mandarins, sweet oranges (*Citrus × sinensis*) and lemons (*Citrus limon*). Distance based clustering enabled the differentiation between red and yellow flashed fruits due to the presence of allele 158 on the locus AC01 in blood oranges. Specific goal of the study was to clarify the identity of 3 local citrus genotypes through observing their relationships within well-established clustering with the rest of genotypes being known citruses. Local orange ‘Domaca Pomorandza’ grouped with other sweet oranges but could not be differentiated from ‘Navelina’ and ‘Skagss Bonanza Navel’. ‘Gorka Pomorandza’ had unique fingerprint and showed to be sour orange. Local lemon ‘Mjesecar’ showed to may be belonging to the Femminello group of lemons. Genetic characterization of local genotypes will serve for cultivar recognition and their future improvement.

Keywords Local genotype · Genetic evaluation · Clustering · Lemon · Orange · Sour orange

Bestimmung von *Citrus*-Genotypen aus Montenegro mittels Mikrosatelliten-Cluster-Analysen

Schlüsselwörter Lokaler Genotyp · Genetische Bewertung · Cluster-Analyse · Zitrone · Orange · Bitterorange

Introduction
Sweet orange, mandarin, pummelo, grapefruit, lemon, lime and other citrus fruits are globally traded either as a fresh or being processed to juice products thus having significant economic importance (Garcia-Lor et al. 2015). Citruses are widely distributed in the tropics and partly in the subtropics and Mediterranean area. Although Montenegro does not represent native habitat of citruses, their cultivation there has been well known and regionally important. Montenegrin Adriatic Sea coastal area (294 km long) is characterized with having the Mediterranean climate and citrus trees started only during the XVI century. Oranges were among the first ones to be introduced for cultivation and orange gardens were mentioned in the old documents from Kotor in 1598 (Plamenac 1978). Nowadays, the largest part of citrus production in Montenegro is based on cultivation of Satsuma mandarins (Radulovic et al. 2005) following with the clementine mandarins, early ripening oranges, lemon Meyer and Kumquat. Those citruses are almost exclusively grafted to the *Poncirus trifoliata* L. Ref. rootstock. In the collection orchard of the Center for Subtropical Cultures (University of Montenegro Biotechnical Faculty) and in private orchards in Bar in Montenegro, there are dozens of citrus genotypes belonging to different species, cul-
vars and hybrids. Classification and evaluation of cultivars and selected clones of citrus are based on morphological traits (Malidzan et al. 2005; Radulovic et al. 2005) while molecular characterization of them is still missing. In this study we focused on genetic identity analysis of 32 citrus genotypes from Montenegro collection. Genetic identity and diversity of citrus germplasm worldwide has been studied for a long time and with many different approaches in order to support selection and breeding of citrus crops. Those studies were challenged by unresolved and controversial citrus phylogenetic history (Velasco and Licciardello 2014) resulting in conflicting taxonomy (Wu et al. 2018) due to the hybridizations between sexually compatible different species during long interbreeding history. Beside recent progress in citrus research by use of sequencing technologies, traditionally diverse molecular markers (RAPDs, RFLPs, ISSRs and SSRs) have been used individually or combined, for citrus identifications, genetic variability assessments, phylogenetic analyses and linkage map constructions (Cai et al. 1994; Fang et al. 1997; Federici et al. 1998; Gulsen and Roose 2001; Krueger and Roose 2003; Sankar and Moore 2001; Siragusa et al. 2006). In this study a set of 11 previously described microsatellite markers were chosen in order to obtain genetic fingerprints of 32 citrus genotypes selected from Montenegro collection. The three (3) local genotypes ‘Domaca Pomorandza’ (orange), ‘Gorka Pomorandza’ (sour orange) and ‘Mjesecar’ (lemon) were of particular interest as they showed to be promising for including into selection and breeding programs. Identity analyses were implemented through two steps: studying duplicates in order to obtain unique fingerprints (I) and clustering of genotypes of interest into groups containing previously described and known citrus (II).

Materials and Methods

Plant Material from Montenegrin Citrus Collection

A total of 32 genotypes that were used for the diversity analysis with SSR markers were obtained from Montenegro citrus collection located at the Center for Subtropical Cultures in Bar (University of Montenegro Biotechnical Faculty). Thirty genotypes were considered as widespread international cultivars belonging to genus Citrus including three genotypes of putative local origin: ‘Domaca Pomorandza’ (Citrus sinensis (L.) Osbeck) (Fig. 1a), ‘Gorka Pomorandza’ (Citrus aurantium L.) (Fig. 1b) and ‘Mjesecar’ (Citrus limon (L.) Burm. f.) (Fig. 1c).

In addition to those, two genotypes were from other genera i.e. kumquat from the genus Fortunella and trifoliate orange from genus Poncirus, the latter one being the most important species for citrus rootstock improvement. The names of genotypes with corresponding species and genus names are listed in Table 1.

DNA Isolation, Microsatellite PCR Amplification and Detection of PCR Fragments

Total genomic DNA was extracted from leaf tissues by modified CTAB method according to Kump and Javornik (1996). The DNA concentration was measured by a fluo-

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**Fig. 1** Appearance of fruits and leaves of promising citrus genotypes from Montenegro. a Orange ‘Domaca Pomorandza’ (Citrus sinensis (L.) Osbeck); b Lemon ‘Mjesecar’ (Citrus limon (L.) Burm. f.); c Sour orange ‘Gorka Pomorandza’ (Citrus aurantium L.)
Table 1  Names of analyzed citrus genotypes from Montenegro collection and their according classification

| Taxon name          | Species                     | Genus       | Horticultural group^a |
|---------------------|-----------------------------|-------------|-----------------------|
| 1. Chahara          | Citrus unshiu Marc          | Citrus      | Satsuma mandarin      |
| 2. Clemapo Delizia  | Tangelo                     | Citrus      | Hybrid = Clementine Comune × Mapo Tangelo |
| 3. Clementine Comune| Citrus reticulata Blanco    | Citrus      | Clementine Mandarin   |
| 4. Clementine Comune SRA 63 | Citrus reticulata Blanco     | Citrus      | Clementine Mandarin   |
| 5. Clementine Comune SRA 89 | Citrus reticulata Blanco     | Citrus      | Clementine Mandarin   |
| 6. Clementine Hernandina V.C.R. | Citrus reticulata Blanco    | Citrus      | Clementine Mandarin   |
| 7. Clementine Marisol | Citrus reticulata Blanco    | Citrus      | Clementine Mandarin   |
| 8. Clementine Spinoso V.C.R. | Citrus reticulata Blanco     | Citrus      | Clementine Mandarin   |
| 9. Domaca Pomorandza | Citrus sinensis (L.) Osbeck | Citrus      | Sweet orange          |
| 10. Femminello Siracusano 2 KR NUC | Citrus limon (L.) Burm. f. | Citrus      | Lemon                 |
| 11. Fortunella      | Fortunella margarita (Lour.) | Fortunella  | Kumquat               |
| 12. Gorka Pomorandza | Citrus aurantium L.         | Citrus      | Sour orange           |
| 13. Kawano Wase     | Citrus unshiu Marc          | Citrus      | Satsuma mandarin      |
| 14. Kawano Wase Clone IV/2 | Citrus unshiu Marc         | Citrus      | Satsuma mandarin      |
| 15. Lane Late NUC 2611 | Citrus sinensis (L.) Osbeck | Citrus      | Navel orange          |
| 16. Mjesecar        | Citrus limon (L.) Burm. f.  | Citrus      | Lemon                 |
| 17. Meyer           | Citrus limon (L.) Burm. f.  | Citrus      | Lemon                 |
| 18. Mooro           | Citrus sinensis (L.) Osbeck | Citrus      | Blood orange          |
| 19. Moro NUC 58-8D-1 | Citrus sinensis (L.) Osbeck | Citrus      | Blood orange          |
| 20. Navelina V.C.R. | Citrus sinensis (L.) Osbeck | Citrus      | Navel orange          |
| 21. Nova            | Tangelo                     | Citrus      | Hybrid = Clementine Comune × Orlando Tangelo |
| 22. Owari           | Citrus unshiu Marc          | Citrus      | Satsuma mandarin      |
| 23. Poncirrus       | Poncirus trifoliata (L.) Raf | Poncirus    | Trifoliate orange     |
| 24. Frimasole       | Mandarin hybrid             | Citrus      | Hybrid = Carvalhais mandarin × Miho Wase |
| 25. Salustiana      | Citrus sinensis (L.) Osbeck | Citrus      | Sweet orange          |
| 26. Satsuma 00 NUC  | Citrus unshiu Marc          | Citrus      | Satsuma mandarin      |
| 27. Skaggs Bonanza Navel | Citrus sinensis (L.) Osbeck | Citrus      | Navel orange          |
| 28. Tacle           | Tangelo                     | Citrus      | Hybrid = Clementine Monreal × Tarocco |
| 29. Tarocco Scire NUC D 2062 | Citrus sinensis (L.) Osbeck | Citrus      | Blood orange          |
| 30. Tarocco Tapi    | Citrus sinensis (L.) Osbeck | Citrus      | Blood orange          |
| 31. Tarocco TDV     | Citrus sinensis (L.) Osbeck | Citrus      | Blood orange          |
| 32. Washington Navel| Citrus sinensis (L.) Osbeck | Citrus      | Navel orange          |

Taxonomy used for the classification of genotypes to the certain groups is mainly relying on to the reference of Swingle and Reece (1967) and only in particular cases (for Citrus unshiu) to the reference of Tanaka (1977)

^aHorticultural group is given based on assignment of citruses as in Curk et al. (2015)

rimeter and samples were diluted with TE buffer to 4 ng/µl. A set of 11 microsatellites (AC01, AG14, CAT01, CT02, CT19-02, CT21 and GT03 as obtained from Citrus maxima (L.) DNA (Barkley et al. 2006); CMS4 and CMS7 as obtained from Citrus sinensis (L.) Osbeck (Ahmad et al. 2003) and TAA15 and TAA33 obtained from a hybrid between Citrus × limonia Osbeck (rangpur lime) and Poncirus trifoliata (L.) Raf. (trifoliate orange) (Kijas et al. 1997)) were studied in independent PCR reactions. The PCR mix was prepared in a total volume of 15 µl containing 20 ng of genomic DNA, 5 × PCR buffer (Promega), 0.2 mM of each of dNTPs (Sigma), 2 mM MgCl₂ (Promega), 0.2 µM of each primer and 0.5 U of GoTaq®DNA polymerase (Promega). The forward primer of each pair was labelled either with 6-FAM, VIC, PET or NED fluorescent dye (Applied Biosystems®). Amplifications of microsatellite loci were done according to the following thermal profile: initial denaturation at 95 °C for 20 s, followed by 38 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min and 30 s and a finale step of 8 min at 72 °C. Four by four PCR reactions differing in dye label were merged by alliquoting 4 µl of each. To one µl of merged PCRs were added
0.5 µl of LIZ 600 size standard and 8.5 µl of Hi-Di formamide. The PCR products were separated by capillary electrophoresis using ABI Prism 3730xl DNA analyzer. Amplified fragments (alleles) were analyzed and sized with Peak Scanner™ Software v 2.0 (Applied Biosystems®) and a microsatellite genetic profile, expressed as digits representing the length of alleles in base pairs, was created for each genotype.

**Analysis of Genetic Diversity and Identity of Local Genotypes**

Microsatellite genetic profile was generated for each analyzed genotypes. Feature of the software package CERVUS version 3.0.7. (Kalinowski et al. 2007) for the identity analysis was used to extract the duplicates by comparing genetic profiles among each pair and calculating the probability of identity based on allele frequencies, for any exact match. Statistical analyses were also computed with the CERVUS software on the unique set of genotypes in order to evaluate polymorphic information content of microsatellite markers on analyzed set of genotypes. The following parameters were calculated for each locus: number of alleles at the locus (Na), observed heterozygosity (Ho), expected heterozygosity (He) and polymorphic information content (PIC).

The patterns of genetic variation were assessed by tree-based methods with the aim to additionally confirm identities of 32 citrus genotypes by visualizing the relationships between them. Features of software DARwin version 6.0.015 (Perrier et al. 2003) were used for the calculations of distance measures between genotypes based on the dissimilarity simple matching coefficient obtained from allelic data and the constructed tree by neighboring method. DARwin was also used to resample the data for the bootstrap analysis with 100 replicates. Model-based clustering was done by software package Structure version 2.3.4 (Pritchard et al. 2000). The web based program STRUCTURE HARVESTER version 0.6.94 (Earl and vonHoldt 2012) was used for the Evanno’s method to derive the number of clusters of individuals (Evanno et al. 2005). Ten runs of STRUCTURE were performed by setting the number of clusters (K) ranging from 2 to 10. Each run consisted of a burn-in period of 200,000 iterations followed by 10⁶ Monte Carlo Markov Chain iterations, assuming an admixture model and correlated allele frequencies. For maximizing similarity over multiple runs on the same data for set number of clusters, matrices were permuted to have the closest match by the use of program CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007). The mean of the permuted matrices across different STRUCTURE runs was used in the program DISTRUCT version 1.1 (Rosenberg 2004) to generate an output for visual representation of the aligned cluster assignments.

**Results**

**Unique Sets of Analyzed Genotypes and Duplicates**

The microsatellite genetic profile of each genotype was obtained from the lengths of amplified fragment sizes presented as numbers of base pairs (Supplemental data). The identity analysis revealed 23 unique genotypes and 5 groups of duplicates. The first group of duplicates consisted of two unshiu mandarins having identical genetic profiles being ‘Kawano Wase’ and ‘Kawano Wase Clone IV/2’ being considered as clones of the same cultivar. Among oranges, three groups of duplicates were recorded: ‘Domaca Pomorandza’ = ‘Navelina’ = ‘Skaqs Bonanza Navel’ (1), ‘Lane Late NUC 26 11’ = ‘Washington Navel’ (2) and ‘Tarocco Scire NUC D 20 16’ = ‘Tarocco Tapi’ = ‘Tarocco TDV’ (3). Within the group of clementine mandarins, four genotypes out of six could not be differentiated: ‘Clementine Comune SRA 63’ = ‘Clementine Comune’ = ‘Clementine Comune SRA 89’ = ‘Clementine Hernandina V.C.R.’.

**Information Statistics of Microsatellite Markers**

A total of 72 different alleles (Na) (Table 2) were observed in analysis of 23 unique genotypes for the 11 microsatellite giving an average number of 6.5 alleles per locus. Locus TAA15 had the highest number of alleles (10) while loci AC01 and CT02 had 4 alleles each (Table 2). CMS4, GT03, TAA15 and CT19-02 were among the most informative in this study having PIC values above 0.6. The observed heterozygosity (Ho) ranged from 0.281 (AC01) to 0.938 (CMS4, CT19-02 and TAA15) with average Ho be-

| Locus | AC01 | AG14 | CAT01 | CMS4 | CMS7 | CT02 | CT19-02 | CT21 | GT03 | TAA15 | TAA33 | Total | Average |
|-------|------|------|-------|------|------|------|---------|------|------|-------|-------|-------|--------|
| Na    | 4    | 9    | 5     | 9    | 6    | 4    | 8       | 5    | 7    | 10    | 5     | 72    | 6.545  |
| Ho    | 0.281 | 0.563 | 0.321 | 0.938 | 0.733 | 0.625 | 0.938   | 0.448 | 0.813 | 0.938 | 0.5   | –     | 0.645  |
| He    | 0.544 | 0.571 | 0.401 | 0.689 | 0.621 | 0.584 | 0.783   | 0.546 | 0.721 | 0.739 | 0.516 | –     | 0.610  |
| PIC   | 0.466 | 0.525 | 0.373 | 0.625 | 0.539 | 0.512 | 0.735   | 0.489 | 0.655 | 0.686 | 0.454 | –     | 0.551  |

*Na* number of alleles per locus, *Ho* observed heterozygosity, *He* expected heterozygosity, *PIC* polymorphic information content, *NE-I Combined non-exclusion probability (identity)*
The expected heterozygosity ranged from 0.401 (CAT01) to 0.783 (CT19-02). The expected heterozygosity had an average value of 0.610 and was somewhat lower than the observed heterozygosity.

**Clustering Analysis**

Based on the microsatellite allelic data dissimilarity, the simple matching coefficient was calculated for the set of all 32 samples and they were clustered using a neighbor-joining method (Fig. 2). The dissimilarity values among the studied genotypes ranged from 0.34 to 0.73. The dendrogram resulted in four clearly separated groups which was also supported by high bootstrap values (>70%): clementine mandarins (100%), sweet and blood oranges (88%), unshiu mandarins (70%) and lemons with one sour orange (92%) (Fig. 2). With structure analysis a highest delta K value (18.659) was obtained when the number of clusters was equal to 4 showing clearly distinguished groups of clementine mandarin, lemon, orange and unshiu mandarin genotypes (Fig. 3) and each genotype was presented in vertical line divided into colored sections where each color was representing one of 4 clusters. The advantage of using different approaches of molecular analyses is well represented for a cluster of oranges where they are divided to two sub-clusters representing blood oranges and yellow oranges due to the presence of allele 158 on the locus AC01 in blood oranges, while these two groups of oranges could not be differentiated by structure analysis probably due to their most recent immediate ancestor and high admixture.

**Clementine and Unshiu Mandarins**

Mandarin is a common name given to a commercial or popular designation of a group of citruses having small and easy-peeling fruits (Garcia-Lor et al. 2015; Wu et al. 2014). Within the clustering analyses, clementine and unshiu mandarins separated each into a different group. This is in accordance with different hybrid origin of Satsumas than clementine mandarins. Namely, although having common...
ancestor, Clementine and Satsuma mandarins are probably separated during evolution by divergent introgression events (Curk et al. 2015).

**Lemons and the Sour Orange**

Local sour orange ‘Gorka Pomorandza’ clustered with 3 lemons (‘Femminello Siracusano 2 KR NUC’; ‘Meyer’ and ‘Mjesecar’) (38% similarity) and separately from the rest of the oranges. This perhaps is depicting the fact that lemons are natural hybrids between one citrus ancestral species *i.e.* citron (*C. medica*) and sour orange (*C. aurantium*) (Carbonell-Caballero et al. 2015; Ollitrault et al. 2012; Wu et al. 2018) while sweet orange and sour orange originated from separated hybridization or interspecific admixture events (Carbonell-Caballero et al. 2015; Wu et al. 2018) i.e. arose from very different breeding programs (Velasco and Licciardello 2014). Lemon cultivars are usually grouped into three types: Sicilian, Verna and Femminello. Local lemon ‘Mjesecar’ showed to be more similar to lemon ‘Femminello Siracusano 2 KR NUC’ (77%) than to ‘Meyer’ (61%). This might be indice to assign ‘Mjesecar’ into the Femminello group of lemons being the most important ones in *e.g.* Italy (Agusti et al. 2014).

**Oranges**

All analyzed oranges clustered together with bootstrap value 85%. Surprisingly, clear differentiation of bloody oranges and yellow oranges was noticed on a dendrogram (Fig. 2) while in other microsatellite studies was reported that differentiation could not be detected (Ahmad et al. 2003; Fang et al. 1997; Hvarleva et al. 2008; Novelli et al. 2006). Montenegrin genotype ‘Domaca Pomorandza’ shared identical microsatellite profile with two other navel oranges ‘Navelina’ and ‘Skagss Bonanza Navel’ despite having different morphological characters (unpublished observations). It was shown earlier that not all sweet oranges could be differentiated by the use of microsatellites (Ahmad et al. 2003; Fang et al. 1997; Hvarleva et al. 2008; Novelli et al. 2006) due to the single ancestor origin and the development of cultivars from the bud mutations (Novelli et al. 2006).

**Conclusions**

Described results are presenting the initial attempt of the molecular evaluation of Montenegro citrus collection. Microsatellite markers proved to be useful in citrus genotype identification through the duplicate elimination and clustering with a set of well-known and previously described cultivars as in case of sour orange ‘Gorka Pomorandza’ and lemon ‘Mjesecar’. For the differentiation of cultivars derived from bud mutations (oranges; Montenegrin local genotype ‘Domaca Pomorandza’) some other technique or combination of techniques should be chosen for describing differences. Successful genetic identification from this study will be used in further wider evaluations and for cit-
rus cultivar recognitions including their future improvement through the selection and breeding.

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**Conflict of interest** L. Bitz, S. Malidzan, N. Stajner, T. Tenhola-Roininen and B. Javornik declare that they have no competing interests.

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