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# Developing Microsatellite Multiplex and Megaplex PCR Systems for High-Throughput Characterization of Breeding Progenies and Linkage Maps Spanning the Apricot (*Prunus armeciaca* L.) Genome

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**Abstract** One hundred and twenty apricot and peach simple sequence repeat (SSR) markers have been used in the molecular characterization of a BC1 apricot progeny of 73 seedlings derived from the cross between the F1 selection "Z506-07" ("Orange Red" × "Currot") and the Spanish cultivar "Currot." To reduce costs and improve the capacity of molecular characterization assays using SSR markers, a series of seven megaplex PCRs containing between six and 20 SSR markers were developed for the molecular characterization of the apricot breeding progeny studied. Amplification was successful in apricot progenitors and in the progeny with 114 of the 120 (95%) SSR markers with a suitable level of polymorphism (1.7 alleles/marker) detected in the BC1 descendants studied. In addition, the implementation of megaplex PCR increased the efficiency and reduced the cost of this type of molecular studies. The implications of these results for apricot-breeding programs and the construction of genetic linkage maps have been also discussed.

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## Introduction

DNA marker technology has become an essential tool for the molecular characterization of plant species. From the end of the 1980s, the utilization of PCR-based markers has increased the opportunities for molecular characterization and mapping of populations in a wide range of plant species including fruit crops. One of the most used PCR-based markers is simple sequence repeat markers (SSR, i.e., microsatellites). This type of markers has been described as the best DNA markers for the assessment of genetic diversity within plant species because of their high polymorphism, abundance, and codominant inheritance (Gupta et al. 1996; Wünsch and Hormaza 2002). In the case of *Prunus* species, hundreds of primer pairs flanking SSRs have been cloned and sequenced in different species including peach, apricot, cherry, and almond (Cipriani et al. 1999, 2001; Testolin et al. 2000; Dirlewanger et al. 2002; Aranzana et al. 2003; Martínez-Gómez et al. 2003a; Dondini et al. 2007). In the international database Genome Database for Rosaceae (www.genome.clemson.edu/gdr), the information on these SSR markers can be compiled. Molecular studies using SSR markers are performed routinely in fruit-breeding programs, including apricot, allowing the characterization of progenitors and the design of new crosses (Hormaza 2002; Zhebentyayeva et al. 2003; Sánchez-Pérez et al. 2005). In addition, further studies using SSR markers in progenies segregating for agronomic traits are being performed for the development of genetic

**Table 1** SSR markers assayed, listed based on the size of the amplified sequence in the original species, and multiplex PCR (indicated with a different shades) performed in the characterization of the apricot breeding progeny

| SSRs | Reference | Annealing Temperature | Original size (bp) | Labelling |
|---|---|---|---|---|
| BPPCT-024 | Dirlewanger et al. 2002 | 57-60 | 96 | 6-FAM |
| AMPA095 | Hagen et al. 2004 | 56 | 100 | NED |
| UDAp-474 | Messina et al. 2004 | 56 | 100 | PET |
| UDP98-406b | Cipriani et al. 1999 | 57-63 | 101 | VIC |
| AMPA118 | Hagen et al. 2004 | 55 | 104 | 6-FAM |
| UDP98-405b | Cipriani et al. 1999 | 57-63 | 104 | NED |
| UDP98-024 | Cipriani et al. 1999 | 57-63 | 105 | PET |
| AMPA124 | Hagen et al. 2004 | 55 | 110 | VIC |
| BPPCT-035C | Dirlewanger et al. 2002 | 57-60 | 113 | 6-FAM |
| UDAp-493 | Messina et al. 2004 | 50 | 113 | NED |
| BPPCT-006C | Dirlewanger et al. 2002 | 57-60 | 117 | PET |
| UDAp-439 | Messina et al. 2004 | 56 | 118 | VIC |
| UDAp-470 | Messina et al. 2004 | 56 | 118 | 6-FAM |
| AMPA116 | Hagen et al. 2004 | 55 | 119 | NED |
| AMPA115 | Hagen et al. 2004 | 55 | 125 | PET |
| UDP98-409b | Cipriani et al. 1999 | 57-63 | 129 | VIC |
| UDP98-412 | Cipriani et al. 1999 | 57-63 | 129 | 6-FAM |
| UDP97-401b | Cipriani et al. 1999 | 57-63 | 130 | NED |
| UDP96-010b | Cipriani et al. 1999 | 57-63 | 131 | PET |
| AMPA122 | Hagen et al. 2004 | 55 | 132 | VIC |
| UDAp-432 | Messina et al. 2004 | 56 | 132 | 6-FAM |
| BPPCT-026 | Dirlewanger et al. 2002 | 57-60 | 134 | NED |
| BPPCT-038 | Dirlewanger et al. 2002 | 57-60 | 135 | PET |
| BPPCT-040 | Dirlewanger et al. 2002 | 57-60 | 135 | VIC |
| UDAp-435 c | Messina et al. 2004 | 50 | 135 | 6-FAM |
| AMPA113 | Hagen et al. 2004 | 55 | 136 | NED |
| UDP97-402b | Cipriani et al. 1999 | 57-63 | 136 | PET |
| UDAp-491 | Messina et al. 2004 | 56 | 140 | VIC |
| UDAp-412 a b | Messina et al. 2004 | 56 | 141 | 6-FAM |
| UDAp-463 | Messina et al. 2004 | 56 | 141 | NED |
| UDP96-003b | Cipriani et al. 1999 | 57-63 | 143 | PET |
| UDP98-021 | Cipriani et al. 1999 | 57-63 | 145 | VIC |
| AMPA110 | Hagen et al. 2004 | 55 | 146 | 6-FAM |
| UDAp-454 | Messina et al. 2004 | 56 | 147 | NED |
| BPPCT-008b | Dirlewanger et al. 2002 | 57-60 | 148 | PET |
| BPPCT-007 | Dirlewanger et al. 2002 | 57-60 | 149 | VIC |
| UDAp-405 | Messina et al. 2004 | 56 | 149 | 6-FAM |
| UDAp-446 | Messina et al. 2004 | 56 | 149 | NED |
| UDAp-462 | Messina et al. 2004 | 50 | 149 | PET |
| UDP97-403b | Cipriani et al. 1999 | 57-63 | 150 | VIC |

maps and markers associated with genes or QTLs involved in the inheritance of the agronomic traits in many *Prunus* species including apricot (Martínez-Gómez et al. 2003a; Dirlewanger et al. 2004; Dondini et al. 2007).

To reduce cost and improve the efficiency and throughput of the molecular characterization assays using PCR-based markers, multiplex PCR, a variant of the PCR in which more than one target sequence is amplified using

**Table 1** (continued)

| SSRs | Reference | Annealing Temperature | Original size (bp) | Labelling |
|---|---|---|---|---|
| UDP98-411 | Cipriani et al. 1999 | 57-63 | 150 | 6-FAM |
| UDAp-486 | Messina et al. 2004 | 56 | 153 | NED |
| BPPCT-039ab | Dirlewanger et al. 2002 | 57-60 | 154 | PET |
| UDAp-473 | Messina et al. 2004 | 56 | 154 | VIC |
| AMPA094 | Hagen et al. 2004 | 55 | 155 | 6-FAM |
| BPPCT-037 | Dirlewanger et al. 2002 | 57-60 | 155 | NED |
| UDP96-005b | Cipriani et al. 1999 | 57-63 | 155 | PET |
| UDAp-415 | Messina et al. 2004 | 56 | 156 | VIC |
| UDAp-437 | Messina et al. 2004 | 56 | 156 | 6-FAM |
| UDAp-489 | Messina et al. 2004 | 56 | 156 | NED |
| UDAp-468 | Messina et al. 2004 | 56 | 157 | PET |
| UDAp-471 | Messina et al. 2004 | 56 | 157 | VIC |
| UDAp-483 | Messina et al. 2004 | 50 | 157 | 6-FAM |
| UDAp-457 | Messina et al. 2004 | 50 | 158 | NED |
| UDAp-410 | Messina et al. 2004 | 56 | 159 | PET |
| UDAp-421 a b | Messina et al. 2004 | 56 | 159 | VIC |
| UDAp-438 | Messina et al. 2004 | 56 | 159 | 6-FAM |
| pchgms5 | Sosinski et al. 2000 | 58 | 160 | NED |
| UDAp-452 | Messina et al. 2004 | 50 | 160 | PET |
| UDAp-458 | Messina et al. 2004 | 56 | 160 | VIC |
| UDAp-466 | Messina et al. 2004 | 56 | 160 | 6-FAM |
| UDAp-406 | Messina et al. 2004 | 56 | 161 | NED |
| UDAp-460 | Messina et al. 2004 | 56 | 161 | PET |
| UDAp-496 | Messina et al. 2004 | 56 | 162 | VIC |
| pchgms2 | Sosinski et al. 2000 | 58 | 163 | 6-FAM |
| UDAp-444 | Messina et al. 2004 | 56 | 163 | NED |
| BPPCT-012 | Dirlewanger et al. 2002 | 57-60 | 164 | PET |
| BPPCT-028 | Dirlewanger et al. 2002 | 57-60 | 164 | VIC |
| UDAp-497 | Messina et al. 2004 | 56 | 166 | 6-FAM |
| UDAp-409 | Messina et al. 2004 | 56 | 167 | NED |
| UDAp-449 | Messina et al. 2004 | 56 | 167 | PET |
| UDAp-456 | Messina et al. 2004 | 56 | 167 | VIC |
| UDAp-451 | Messina et al. 2004 | 50 | 168 | 6-FAM |
| UDAp-418 | Messina et al. 2004 | 56 | 169 | NED |
| BPPCT-009 | Dirlewanger et al. 2002 | 57-60 | 171 | PET |
| UDAp-479 | Messina et al. 2004 | 56 | 171 | VIC |
| BPPCT-011 | Dirlewanger et al. 2002 | 57-60 | 172 | 6-FAM |
| UDAp-461 | Messina et al. 2004 | 56 | 173 | NED |
| AMPA096 | Hagen et al. 2004 | 55 | 174 | PET |
| BPPCT-017 | Dirlewanger et al. 2002 | 57-60 | 174 | VIC |

more than one pair of primers (and usually less than four), is being assayed in molecular studies (Sánchez-Pérez et al. 2004; Hayden et al. 2008; Patocchi et al. 2009; Pascal et al. 2009). On the other hand, megaplex PCR is a very recent and robust technology for highly multiplexed amplification of specific DNA sequences. It uses target-specific pairs of PCR primers (more than four) that are physically separated (Meuzelaar et al. 2007). To date, this methodology has not

**Table 1** (continued)

| SSRs | Reference | Annealing Temperature | Original size (bp) | Labelling |
|---|---|---|---|---|
| pchgms4 | Sosinski et al. 2000 | 58 | 174 | 6-FAM |
| BPPCT-030 | Dirlewanger et al. 2002 | 57-60 | 175 | NED |
| UDAp-420 | Messina et al. 2004 | 56 | 175 | PET |
| UDAp-485 | Messina et al. 2004 | 56 | 177 | VIC |
| UDAp-413 | Messina et al. 2004 | 56 | 179 | 6-FAM |
| UDAp-424 | Messina et al. 2004 | 50 | 179 | NED |
| AMPA107 | Hagen et al. 2004 | 51 | 180 | PET |
| BPPCT-033B | Dirlewanger et al. 2002 | 57-60 | 180 | VIC |
| BPPCT-013 | Dirlewanger et al. 2002 | 57-60 | 183 | 6-FAM |
| AMPA101 | Hagen et al. 2004 | 55 | 188 | NED |
| UDAp-407 | Messina et al. 2004 | 56 | 188 | PET |
| UDAp-465 | Messina et al. 2004 | 56 | 188 | VIC |
| UDAp-469 | Messina et al. 2004 | 50 | 188 | 6-FAM |
| UDAp-430 | Messina et al. 2004 | 56 | 189 | NED |
| UDAp-450 | Messina et al. 2004 | 50 | 189 | PET |
| AMPA105 | Hagen et al. 2004 | 55 | 191 | VIC |
| UDAp-422 | Messina et al. 2004 | 50 | 191 | 6-FAM |
| UDAp-423 | Messina et al. 2004 | 56 | 192 | NED |
| UDAp-472 | Messina et al. 2004 | 56 | 192 | PET |
| BPPCT-019C | Dirlewanger et al. 2002 | 57-60 | 194 | VIC |
| BPPCT-025 | Dirlewanger et al. 2002 | 57-60 | 197 | 6-FAM |
| BPPCT-004 | Dirlewanger et al. 2002 | 57-60 | 200 | NED |
| BPPCT-020B | Dirlewanger et al. 2002 | 57-60 | 200 | PET |
| UDAp-401 | Messina et al. 2004 | 56 | 201 | VIC |
| UDAp-436 a b | Messina et al. 2004 | 56 | 202 | 6-FAM |
| AMPA109 | Hagen et al. 2004 | 55 | 204 | NED |
| UDAp-503 | Messina et al. 2004 | 56 | 204 | PET |
| UDAp-467 | Messina et al. 2004 | 56 | 211 | VIC |
| AMPA100 | Hagen et al. 2004 | 55 | 215 | 6-FAM |
| BPPCT-014 | Dirlewanger et al. 2002 | 57-60 | 215 | NED |
| BPPCT-041 | Dirlewanger et al. 2002 | 57-60 | 220 | PET |
| pchcms3 | Sosinski et al. 2000 | | 220 | VIC |
| BPPCT-018B | Dirlewanger et al. 2002 | 57-60 | 222 | 6-FAM |
| AMPA112 | Hagen et al. 2004 | 55 | 223 | NED |
| pchcms4 | Sosinski et al. 2000 | 58 | 225 | PET |
| UDAp-431ab | Messina et al. 2004 | 56 | 227 | VIC |
| BPPCT-002 | Dirlewanger et al. 2002 | 57-60 | 229 | 6-FAM |
| UDAp-441 | Messina et al. 2004 | 56 | 237 | NED |
| UDAp-499 | Messina et al. 2004 | 56 | 248 | PET |
| BPPCT-027ab | Dirlewanger et al. 2002 | 57-60 | 249 | VIC |

been applied in *Prunus* species. The most important advantage of this megaplex PCR system is the choice of many oligonucleotide primers to improve the speed of the molecular characterization assays and to facilitate the automation of this process also reducing the cost.

The objective of this work was the development of microsatellite multiplex and megaplex PCR systems for high-throughput characterization of apricot (*Prunus armeciaca* L.) progenies and development of linkage maps spanning most of genome.

**Table 2** Megaplex, multiplex and single PCR (indicated with different shades) designed for the molecular characterization of the apricot breeding progeny using different SSR markers and range of the size of the resulted PCR products

| SSRs | Labelling | Range (bp) Min | Max |
|---|---|---|---|
| Megaplex 1 | | | |
| AMPA095 | NED | 85 | 142 |
| UDP98406 | VIC | 89 | 128 |
| UDAp470 | 6-FAM | 84 | 118 |
| BPPCTO38 | PET | 80 | 142 |
| AMPA110 | 6-FAM | 126 | 146 |
| BPPCT007 | VIC | 135 | 158 |
| UDAp405 | 6-FAM | 164 | 164 |
| UDAp460 | PET | 148 | 165 |
| BPPCT028 | VIC | 169 | 170 |
| UDAP418 | NED | 160 | 168 |
| BPPCT011 | 6-FAM | 173 | 185 |
| Pchgms4 | 6-FAM | 153 | 153 |
| UDAp485 | VIC | 181 | 211 |
| UDAp430 | NED | 176 | 198 |
| UDAp450 | PET | 185 | 217 |
| AMPA112 | NED | 204 | 222 |
| UDAp499 | PET | 226 | 249 |
| PCT027 | VIC | 238 | 242 |
| Megaplex 2 | | | |
| BPPCT-024 | 6-FAM | 94 | 137 |
| BPPCT-006C | PET | 80 | 143 |
| UDAp-439 | VIC | 98 | 135 |
| BPPCT-026 | NED | 96 | 141 |
| UDAp-415 | VIC | 149 | 161 |
| UDAp-437 | 6-FAM | 146 | 171 |
| pchgms5 | NED | 162 | 173 |
| UDAp-452 | PET | 154 | 164 |
| UDAp-449 | PET | 172 | 173 |
| UDAp-413 | 6-FAM | 178 | 288 |
| UDAp-424 | NED | 185 | 341 |
| UDAp-401 | VIC | 171 | 214 |
| UDAp-503 | PET | 185 | 220 |
| pchcms4 | PET | 232 | 236 |
| Megaplex 4 | | | |
| UDAp-493 | NED | 97 | 135 |
| AMPA115 | PET | 83 | 127 |
| UDAp-491 | VIC | 139 | 159 |
| BPPCT-039 | PET | 133 | 150 |
| UDAp-421 | VIC | 112 | 133 |
| pchgms2 | 6-FAM | 160 | 169 |
| UDAp-497 | 6-FAM | 144 | 144 |
| UDAp-420 | PET | 161 | 175 |
| BPPCT-013 | 6-FAM | 140 | 140 |
| UDAp-422 | 6-FAM | 181 | 198 |
| UDAp-423 | NED | 171 | 302 |
| BPPCT-019C | VIC | 214 | 240 |
| BPPCT-025 | NED | 148 | 150 |
| UDAp-467 | VIC | 186 | 202 |
| Megaplex 3 | | | |
| UDP96-010b | PET | 83 | 131 |
| UDAp-432 | 6-FAM | 96 | 128 |
| BPPCT-040 | VIC | 108 | 135 |
| UDP97-402b | PET | 141 | 146 |
| UDAp-463 | NED | 110 | 122 |
| UDAp-446 | NED | 131 | 153 |
| UDP98-411 | 6-FAM | 167 | 179 |
| UDAp-438 | 6-FAM | 139 | 139 |
| UDAp-466 | 6-FAM | 147 | 162 |
| UDAp-456 | VIC | 151 | 169 |
| UDAp-461 | NED | 162 | 175 |
| BPPCT-017 | VIC | 174 | 186 |
| UDAp-407 | PET | 193 | 193 |
| AMPA105 | VIC | 198 | 217 |
| UDAp-472 | PET | 156 | 184 |
| BPPCT-004 | NED | 197 | 203 |
| BPPCT-020B | PET | 198 | 198 |
| AMPA100 | 6-FAM | 186 | 217 |
| BPPCT-014 | NED | 186 | 186 |
| BPPCT-041 | PET | 215 | 215 |
| Megaplex 5 | | | |
| AMPA118 | 6-FAM | 97 | 108 |
| UDAp-454 | NED | 97 | 161 |
| BPPCT-008b | PET | 92 | 128 |
| UDAp-462 | PET | 147 | 153 |
| UDP97-403b | VIC | 118 | 118 |
| UDAp-451 | 6-FAM | 131 | 167 |
| AMPA107 | PET | 172 | 242 |
| BPPCT-033b | VIC | 126 | 165 |
| AMPA101 | NED | 188 | 188 |
| AMPA109 | NED | 199 | 205 |
| BPPCT-002 | 6-FAM | 188 | 195 |
| Megaplex 6 | | | |
| UDP98-405b | NED | 104 | 148 |
| UDP98-412 | 6-FAM | 104 | 116 |
| UDP96-003b | PET | 94 | 106 |
| UDAp-473 | VIC | 115 | 145 |
| AMPA094 | 6-FAM | 132 | 154 |
| UDP96-005b | PET | 119 | 146 |
| UDAp-479 | VIC | 151 | 196 |
| Megaplex 7 | | | |
| UDAp-474 | PET | 99 | 100 |
| BPPCT-035C | 6-FAM | 96 | 148 |
| UDAp-471 | VIC | 121 | 147 |
| UDAp-406 | NED | 105 | 179 |
| UDAp-496 | VIC | 156 | 192 |
| BPPCT-012 | PET | 132 | 169 |
| Multiplex 8 | | | |
| UDP98-024 | PET | 91 | 146 |
| AMPA124 | VIC | 108 | 156 |
| UDAp-444 | NED | 108 | 170 |
| UDAp-436ab | 6-FAM | 103 | 262 |
| Multiplex 9 | | | |
| UDP97-401b | NED | 109 | 142 |
| UDAp-412ab | 6-FAM | 132 | 152 |
| UDAp-410 | PET | 122 | 149 |
| UDAp-465 | VIC | 119 | 173 |
| Multiplex 10 | | | |
| UDAp-468 | PET | 146 | 168 |
| UDAp-483 | 6-FAM | 133 | 160 |
| UDAp-458 | VIC | 127 | 170 |
| UDAp-409 | NED | 109 | 160 |
| Multiplex 11 | | | |
| UDP97-401b | NED | 112 | 136 |
| UDP98-409b | VIC | 132 | 161 |
| UDAp-469 | 6-FAM | 141 | 217 |
| Multiplex 12 | | | |
| AMPA122 | VIC | 132 | 161 |
| UDAp-486 | NED | 115 | 154 |
| Multiplex 13 | | | |
| UDP98-021 | VIC | 153 | 153 |
| BPPCT-037 | NED | 115 | 161 |
| Single 14 | | | |
| UDAp-441 | NED | 120 | 322 |
| Single 15 | | | |
| UDAp-489 | NED | 132 | 166 |
| Single 16 | | | |
| UDAp-457 | NED | 133 | 166 |
| Single 17 | | | |
| AMPA113 | NED | 134 | 161 |
| Single 18 | | | |
| BPPCT-030 | NED | 136 | 146 |
| No amplification | | | |
| UDAp-435c | 6-FAM | 0 | 0 |
| BPPCT-018b | 6-FAM | 0 | 0 |
| pchcms3 | VIC | 0 | 0 |
| UDAp-431ab | VIC | 0 | 0 |
| BPPCT-009 | PET | 0 | 0 |
| AMPA096 | PET | 0 | 0 |

## Material and Methods

### Plant Material

The plant material assayed included the North American apricot cultivar "Orange Red" ®, the Spanish "Currot," and a BC1 progeny of 73 seedlings derived from the cross between the F1 selection "Z506-07" ("Orange Red" × "Currot") and the Spanish cultivar "Currot" made in 2001. All genotypes were cultivated in the same experimental orchard in Calasparra, in the Region of Murcia, Southeast Spain (lat. 37° N, long. 1° W).

### DNA Isolation

Total genomic DNA was isolated using the procedure described by Doyle and Doyle (1987). Approximately 50 mg of young leaves were ground in a 1.5-ml Eppendorf tube with 750 µl of CTAB extraction buffer (100 mM Tris–HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% $NaHSO_3$). Samples were incubated at 65°C for 20 min, mixed with an equal volume of 24:1 chloroform–isoamyl alcohol, and centrifuged at 6,000×*g* for 20 min. The upper phase was recovered and mixed with an equal volume of isopropanol at −20°C. The nucleic acid precipitated was washed in 400 µl of 10 mM $NH_4Ac$ in 76% ethanol, dried, resuspended in 50 µl of TE (10 mM Tris–HCl, 0.1 mM EDTA, pH8.0), and incubated with 0.5 µg of RNase A at 37°C for 30 min to digest RNA.

### SSR Analysis

Extracted apricot genomic DNA was PCR-amplified using 120 pairs of primers flanking SSR sequences, previously cloned, and sequenced in peach (44 SSRs) (Cipriani et al. 1999; Sosinski et al. 2000; Dirlewanger et al. 2002) and apricot (76 SSRs) (Hagen et al. 2004; Messina et al. 2004) (Table 1). SSR-PCR amplifications were performed in 5 µL reaction mixture containing 2.5 µL of commercial Taq PCR Master Mix Kit (Qiagen, Hilden, Germany), 20 nM of each forward primer labeled with a fluorescent dye [6-FAM (blue); VIC (green); NED (yellow); PET (red)] (Applied Biosystems, Foster City, CA), 20 nM of each unlabelled reverse primer, 5 ng of genomic DNA and 1 µL of $dH_2O$. Amplification was performed for 40 cycles at 94°C for 30 s, 58°C for 1 min and 30 s, and 72°C for 1 min, for denaturation, annealing, and primer extension, respectively.

### Multiplex and Megaplex PCR Development

Initially, 30 multiplex PCR using four SSR markers were designed (Table 1). Each SSR marker was labeled with one of the four dyes available. These SSR markers were applied in the progenitors and in several individuals of the progeny to check the size of the amplified bands in our apricot genotypes. These results were applied in the development of megaplex PCR using more than four SSR markers paying attention to the size of the amplification products and the dye of the SSR.

### DNA Fragment Analysis

Amplified PCR products were separated and analyzed on an automated capillary sequencer AB 3130x (Applied Biosystems) along GeneScanT 500 RoxT (-250) internal size standard. Allele sizes were scored using GeneMapper V. 4.0 (Applied Biosystem).

### Map Construction

A framework genetic map was constructed using Join Map v. 3.0 software (van Ooijen and Voorrips 2001) by using the CP population type. Linkage groups were established with a minimal LOD of 4.0.

## Results and Discussion

Amplifications were successful in apricot progenitors and in the progeny with 114 of the 120 (95%) SSR markers with a 69 % (79 out of 114 markers) polymorphism detected (number of polymorphic bands ranged from 1 to 4 depending on the codominant nature of the maker) in the apricot BC1 descendants studied. In the case of the SSR markers developed in apricot, 96% of these markers were successfully amplified in the progenitors ("Z506-07" and "Currot") and in the BC1 progeny with the exception of the UDAp-435c, UDAp-431ab (Messina et al. 2004), and AMPA096 (Hagen et al. 2004) (Table 2). SSR markers showed a size ranging between 83 and 242 bp (Table 2). For the majority of the markers, the allele size range matched the one initially described by the authors who developed these markers in both peach (Cipriani et al. 1999; Sosinski et al. 2000; Dirlewanger et al. 2002) and apricot (Hagen et al. 2004; Messina et al. 2004).

A series of seven megaplex PCRs containing between six and 20 SSR markers were assembled for the molecular characterization of the apricot breeding progeny (Table 2). These megaplex PCRs were designed according to the size obtained in our apricot materials (between 83 and 242 bp) by combining the different dyes labeling the SSR markers assayed. In the combination of these SSR markers through megaplex PCR, a minimum interval of 5 bp was kept between the SSR markers with the same labeling to avoid band misidentification (Table 2). Ninety-one of the 120 SSR markers assayed were used in only seven PCR reactions corresponding with the seven megaplexes. Sixty of these 91 markers amplified in the studied progeny and their polymorphism were evaluated for use in the construction of the genetic linkage map. No problems were found in the PCR amplification because of the location of SSR markers in the same PCR reaction. The percentage of markers amplified in the progeny in each megaplex ranged from 100% in the case of megaplex 6 to 28.57% in the megaplex 7 with a mean value of 66% (Table 3). In megaplex 1, for example, a total of 16 markers were amplified in the same PCR (Fig. 1).

Acquired marker data were used to construct the preliminary map of "Z506-07" and "Currot" (Fig. 2). This genetic

**Table 3** Amplification of SSRs in each megaplex PCR

| Megaplex | Number of Primers per megaplex | Number of markers amplified in the progeny | Percent (%) |
|---|---|---|---|
| 1 | 18 | 16 | 88.89 |
| 2 | 14 | 8 | 57.14 |
| 3 | 20 | 11 | 55.00 |
| 4 | 14 | 10 | 71.43 |
| 5 | 11 | 6 | 54.55 |
| 6 | 7 | 7 | 100.00 |
| 7 | 7 | 2 | 28.57 |
| Mean | 13 | 8.57 | 65.93 |
| Total | 91 | 60 | – |

map includes 37 and 29 SSRs, for "Z506-07" and "Currot," respectively (Table 4). The maps cover 240.8 and 226.3 cM for "Z506-07" and "Currot," respectively (Fig. 2), spanning about 45% and 37% of the apricot genome, compared with the *Prunus* reference map (522 cM) (Aranzana et al. 2003; Dirlewanger et al. 2004). Considering both maps together, the SSR distribution was relatively uniform with the exception of linkage group 6. The number of markers per LG varied from none (LGs 6, 7, 8 in "Currot") to nine (LG1 in "Z506-07"; Table 4). The largest LG obtained were 90.0 and 52.9 cM for "Z506-07" and "Currot," respectively.

In this study, a large set of highly polymorphic SSR markers has been identified that are well distributed over the apricot genome. Overall, the results showed a high level of polymorphism in our apricot BC1 population. DNA fingerprints obtained from the amplification of SSR markers are of great importance for different purposes including the certification program to protect new releases from breeding programs, particularly in the cases of species, such as apricot, that are vegetatively propagated (Sánchez-Pérez et al. 2005) or the development of genetic linkage maps and markers associated with genes or QTLs (Dirlewanger et al. 2004). Our results also confirmed the well-described transferability of SSR markers between *Prunus* species (Cipriani et al. 1999; Sosinski et al. 2000; Martínez-Gómez et al. 2003b, c; Zhebentyayeva et al. 2003; Arús et al. 2006): Forty-one of the 44 (93%) SSR markers initially developed in peach amplified successfully in the apricot progeny assayed.

One of the most important applications of this new methodology is to ease the development of genetic linkage maps. The location of the SSR markers mapped in this study was similar to those previously reported by Aranzana et al. (2003) and Dondini et al. (2007). Our results showed a high level of colienarity between *Prunus* species and confirmed a high level of transferability of the markers, as already described by Dirlewanger et al. (2004) and Arús et al. (2006). This homology among *Prunus* species partly explains the low level of breeding barriers to interspecific gene introgression and highlights the opportunity for successful gene transfer between closely related species (Martínez-Gómez et al. 2003c).

The development of the megaplex PCR greatly increased the efficiency and reduced the cost involved in the implementation of this type of molecular characterization studies. This efficiency is derived primarily from the high number of markers amplied in a single reaction. The organization of SSR markers in sets of megaplex PCR also increased the

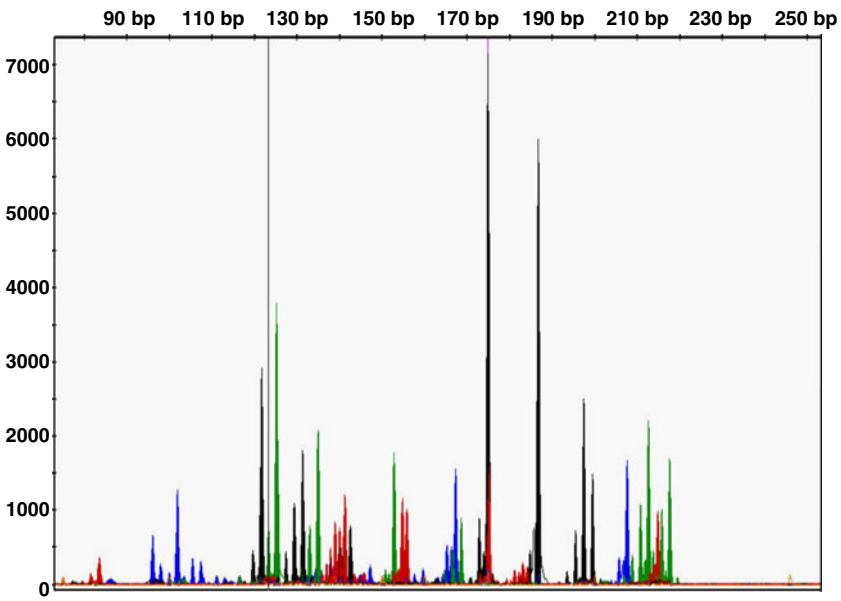


**Fig. 1** Distribution of amplified products in the megaplex PCR 1 after the analysis with the automated capillary electrophoresis. The observed peaks [*blue* (6-FAM dye), *black* (NED), *red* (PET), and *green* (VIC)] correspond to the different SSR markers included in the megaplex

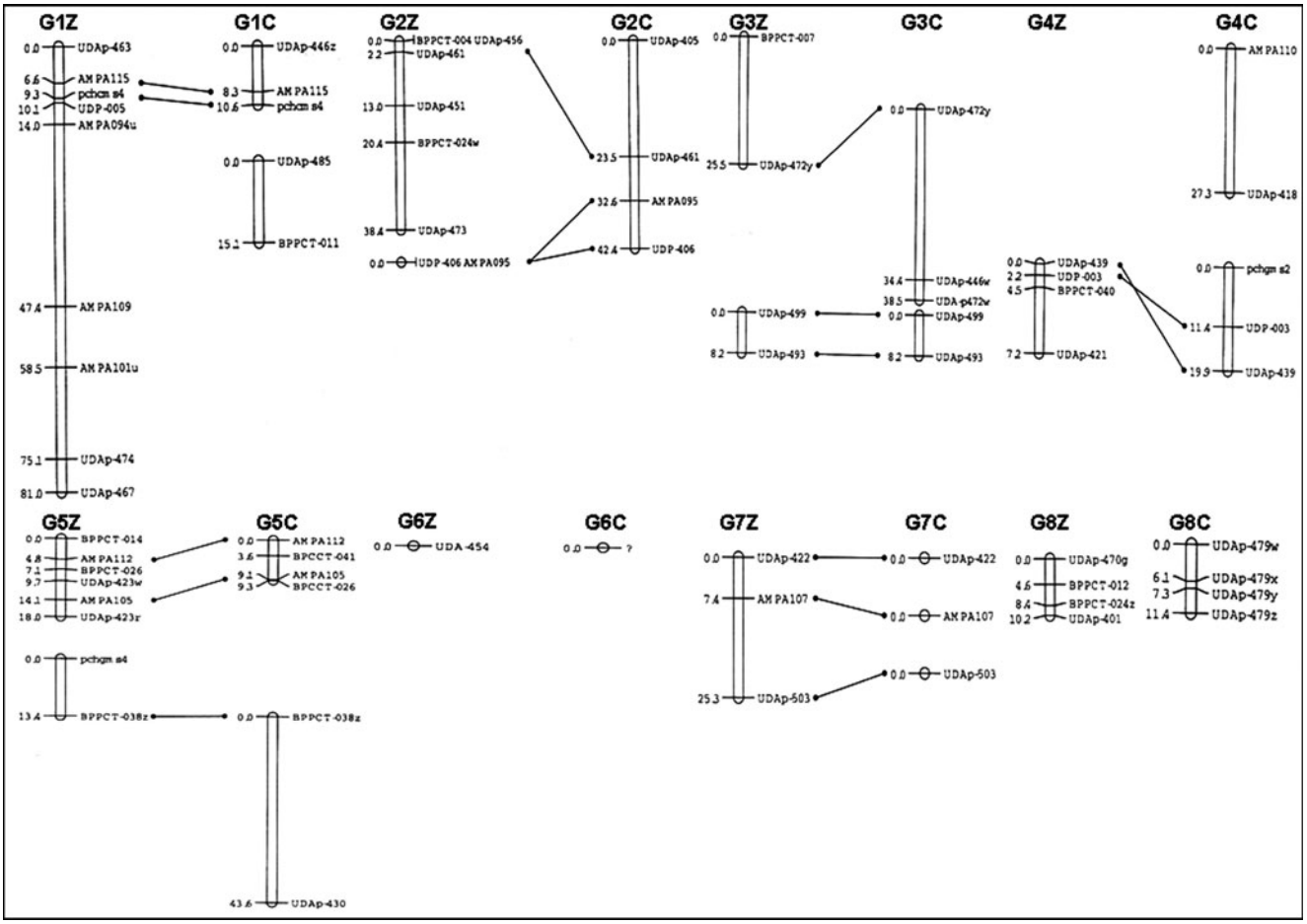


**Fig. 2** Comparison of molecular linkage maps of "Z506-07" (*Z*) and "Currot" (*C*) obtained with 37 and 29 SSRs, respectively, constructed using JOINMAP after the application of seven megaplex PCR including the evaluation of 91 different SSRs

efficiency and reduced the cost of the development of genetic linkage maps in *Prunus* species. The described set of SSR markers included in megaplex 2 that amplified the highest number of SSR markers, exemplified the possibility of the analysis of 18 SSR markers in only one reaction. The price of labeling each forward primer is pretty high, as well as fragment analysis using automated capillary sequencer, making the results of this work even more important since multiplexing of up to 18 markers in one single reaction can significantly reduce the time and cost in development of a

**Table 4** Number of mapped SSR markers, amplified but not mapped (between parentheses), size of the LGs, and marker density (mean distance in cM) in the two linkage maps of "Z506-07" and "Currot"

| Linkage group | Number of SSR markers ('Z506-07') | Size of LG (cM) | Marker density (cM) | Number of SSR markers ('Currot') | Size of LG (cM) | Marker density (cM) |
|---|---|---|---|---|---|---|
| 1 | 9 | 90.0 | 10.00 | 5 | 25.7 | 5.14 |
| 2 | 5+(1) | 38.4 | 7.68 | 4 | 42.4 | 10.60 |
| 3 | 4 | 33.7 | 8.43 | 5 | 46.7 | 9.34 |
| 4 | 4 | 17.2 | 4.30 | 5 | 47.2 | 9.44 |
| 5 | 8 | 18.0 | 2.25 | 6 | 52.9 | 8.82 |
| 6 | (1) | 0 | – | – | – | – |
| 7 | 3 | 25.3 | 8.43 | (3) | – | – |
| 8 | 4 | 18.2 | 4.55 | – | – | – |
| Unknown | – | – | | 4 | 11.4 | 2.85 |
| Total | 37 | 240.8 | 6.52 | 29 | 226.3 | 7.70 |

new genetic map. These results indicate a higher efficiency in comparison with the multiplex PCR developed recently for the analysis of SSR markers in apple (Patocchi et al. 2009) and *Populus* (Pascal et al. 2009).

This work illustrated the possibility of analyzing 20 SSR markers, within initial set of 120 markers with a range of 92–249 bp, in only one reaction. Seeing that the 120 markers set had not been tested in our population prior to selecting the markers, the optimization of the process can be further increased if the exact size of the amplified product of the markers in the progeny is known in advance. Thus, the choice and grouping of primers in the megaplex could be more accurate. Moreover, the higher the range of the amplified sequences, the more markers could be included in a single megaplex. The optimization and use of the megaplex can open new dimensions in the multifunctional use of microsatellites for breeders and geneticists, multiplying the efficiency and significantly reducing the cost of the analysis.

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