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## Validation of criteria for the selection of AFLP markers to assess the genetic variation of a breeders' collection of evergreen azaleas

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Abstract Fluorescent AFLP and automated data analysis were employed to assess the genetic conformity within a breeders' collection of evergreen azaleas. The study included 75 genotypes of Belgian pot azaleas (Rhododendron simsii Planch. hybrids), Kurume and Hirado azaleas and wild ancestor species from the *Tsutsusi* subgenus. Fluorescent detection and addition of an internal size standard to each lane enabled the automated scoring of each fragment arising from a single AFLP primer combination (PC). The use of three PCs generated an initial data set with a total of 648 fragments ranging from 70 bp to 450 bp. Different marker selection thresholds for average fluorescent signal intensity and marker frequency were used to create eight extra restricted data subsets. Pairwise plant genetic similarity was calculated for the nine data sets using the Simple Matching coefficient (symmetrical, including double-zeros) and Jaccard coefficient (asymmetrical, excluding double zeros). The averages, the ranges and the correlation to one other (Mantel analysis) were compared for the obtained similarity matrices. This revealed the sensitivity of ordinations obtained by both similarity coefficients for the presence of weak or intensive markers or for the degree of polymorphism of the markers. For 34 cultivars, pedigree information (at maximum to the fifth ancestor generation) was available. Genetic similarity by descent

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Faculty of Agricultural and Applied Biological Sciences – RUG, Coupure links 653, 9000 Gent, Belgium (kinship coefficient) was turned into a genetic distance and correlated to the genetic conformity, as revealed by the different selections of AFLP markers (Mantel analysis). Use of a Simple Matching coefficient with no or moderate selection to signal intensity and excluding rare and abundant markers gave the best correlation with pedigree. Finally, the ordination of the studied genotypes by means of dendrograms and principal co-ordinate analysis was confronted with known or accepted relationships based on geographical origin, parentage and morphological characters. Genotypes could be assigned to three distinct groups: pot azaleas, Kurume azaleas and Hirado azaleas. Wild ancestor species appeared to be more related to the Japanese azaleas. Intermediate cultivars could be typified as crossings with Kurume or Hirado azaleas or with wild species.

**Key words** Similarity Coefficients · Pedigree · Kinship · *Rhododendron simsii* · Mantel's test

## Introduction

Traditionally, plant breeders have always evaluated plant origins and pedigrees as an extra indication of the characteristics that were acquired by a new genotype. With the advent of molecular marker techniques, new tools that have the capacity to reveal (dis)similarities in the genome of related plant species or cultivars directly became available. Polymerase chain reaction (PCR)-based multilocus fingerprinting techniques for which no prior DNA sequence information is required, like randomly amplified polymorphic DNA (RAPD) or amplified fragment length polymorphism (AFLP), have in particular facilitated this considerably. The AFLP technique, based on the selective PCR amplification of restriction fragments from a total restriction digest of genomic DNA (Vos et al. 1995), often yields several DNA polymorphisms amplified by one primer combination. Because of its reproducibility and the high data output per reaction, AFLP is being used more and more as a fast and reliable

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5     Ademursaki     Hirado       7     Concinan     Hirado       7     Concinan     Hirado       9     Kirin     Kurume       9     Kirin     Kurume       11     TRex     Kurume       12     "Directur Van Stycken     Kurume       13     Palestrim     Kurume       21     Gilber Mills     Kurume       22     Hac Dambe     Kurume       3     Palestrim     Kurume       4     Stevartstownian     Kurume       4     Stevartstownian     Kurume       21     "Apollo     Pot azalea       22     "Apollo     Pot azalea       23     "Ademits Glocke     Pot azalea       24     Madiame Perick     Pot azalea       25     "Laura Ashisp'     Pot azalea       26     Niobe     Pot azalea       27     "Advents Glocke     Pot azalea       28     Edicide de Edigiue     Pot azalea       29     "Piak Dean     Pot azalea       20     "Continese de Kerchove     Pot azalea       21     "Advents Glocke     Pot azalea       23     "Roue Leopold     Pot azalea       24     Tauruon     Pot azalea       25<	Number <sup>a</sup>	Variety	Туре	Parentage <sup>b, c</sup>
6     "Heive-no-hikari     Hirado       7     Concina     Hirado       8     Omarasaki     Hirado       9     'Kirin     Kurume       10     Thira tear Yan Stycken     Kurume       11     Ohne tear Yan Stycken     Kurume       12     Diactear Yan Stycken     Kurume       13     Cheops     Noris-azalea       14     Gilbert Mullic     Kurume       25     Bue Danube     Kurume       26     Hevartstownian     Pot azalea       27     'Apolin     Pot azalea       28     Hordor Wolters     Pot azalea       29     'Profor Wolters     Pot azalea       20     'Profor Wolters     Pot azalea       21     'Apolin     Pot azalea       22     'Profor Wolters     Pot azalea       23     'Profor Wolters     Pot azalea       24     'Profor Wolters     Pot azalea       25     'Laara Ashley     Pot azalea       26     Niche     Pot azalea       27     'Adventyllocke     Pot azalea       28     Etolie de Belgique     Pot azalea       29     'Prak Scheeme     Pot azalea       30     'Coamtese de Kerchove     Pot azalea       31	5	Ademurasaki	Hirado	
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11     Rex     Kurume     Huomayox,Ninn       12     Directeur Van Slycken     Kurume     Excelsor-Rex       13     Gibert Multe     Kurume     Excelsor-Rex       34     Breatstina     Kurume     Excelsor-Rex       35     Palestrina     Kurume     Excelsor-Rex       36     Breatstrina     Kurume     John T.D. Lewelynx/Madame Royer       20     "Arenia"     Pot azalea     John T.D. Lewelynx/Madame Royer       21     "Apolio     Pot azalea     John T.D. Lewelynx/Madame Royer       22     "Arenia"     Pot azalea     Mevrouw Jozef Heursel*xHerrmann's Superbace       23     "Professor Wolters     Pot azalea     Pot azalea       24     Madame Petrick     Pot azalea     Pot azalea       25     "Laura Ashtey     Pot azalea     Put science       26     Niobe     Pot azalea     Put science       27     "Adventsglocke     Pot azalea     Put science       28     Exicito e belgique     Pot azalea     Put azalea       29     "Combosen" cerchove     Pot azalea     Put science       20     "Poit azalea     Herzog Adoff von NasaavAmoenum       30     "Poit azalea     Herzog Adoff von NasaavAmoenum       311     "Fures     Pot azalea	9	*Kirin	Kurume	····
12     Directeur Van Stycken     Kurume     Excelsior>Rex       15     Cheops     Noria-zulea       1     Gilbert Mulie     Kurume       2     Blue Danube     Kurume       4     Professor Wolters     Pot azalea       20     'Vervaenena     Pot azalea       21     'Avenir     Pot azalea       22     'Avenir     Pot azalea       23     'Professor Wolters     Pot azalea       24     'Madame Petrick     Pot azalea       25     'Laura Ashley     Pot azalea       26     Niobe     Pot azalea       27     'Avenir     Pot azalea       28     Eloide de Belgique     Pot azalea       29     'Pink Dream     Pot azalea       20     'Comtess de Kerchove     Pot azalea       21     'Rai Loopold     Pot azalea       23     'Roi Loopold     Pot azalea       24     'Madame Petre     Pot azalea       25     'Laure Freén     Pot azalea       26     Nibe     Pot azalea       27     'Avensjoicke     Pot azalea       28     Eloide de Belgique     Pot azalea       29     'Pink Dream     Pot azalea       20     'Roi Loopold     Pot azalea	11	*Rex	Kurume	Hinomayo×Kirin
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26     Niobe     Pot azalea       27     'Adventsjocke     Pot azalea       28     Etoile de Belgique     Pot azalea       29     'Pink Dream'     Pot azalea       30     'Comtesse de Kerchove     Pot azalea       31     'Paul Schaeme     Pot azalea       32     Roi Leopold     Pot azalea       33     'Hexe     Pot azalea       34     'Euratom     Pot azalea       35     'Kuu Erwén     Pot azalea       36     'Reinhold Ambrosius     Pot azalea       37     'Elcaireur     Pot azalea       38     'Elsa Kaerger     Pot azalea       39     'Ambrosiana     Pot azalea       41     Perfe de Swijnaerde     Pot azalea       42     'Tempérance     Pot azalea       43     'Coolestine     Pot azalea       44     Dame Blanche     Pot azalea       45     'Glaser Nr. 10     Pot azalea       46     'Madame PB. Van Acker     Pot azalea       47     Tamira     Pot azalea       48     Gloire de Saint Georges     Pot azalea       51     'Hellmut Vogel     Pot azalea       52     'Mevrouw Marcel Wanbelle     Pot azalea       53     Doctor Heimann     Pot a	25	*Laura Ashley	Pot azalea	Meyrouw Jozef Heursel <sup>c</sup> ×Herrmann's Superba <sup>c</sup>
27     ''Adventsglocke     Pot azalea     Paul Schaeme×Fritz Sander       28     Etoile de Belgique     Pot azalea       29     'Pink Dream     Pot azalea       30     'Comtesse de Kerchove     Pot azalea       31     'Paul Schaeme     Pot azalea       32     'Roi Loopold     Pot azalea       33     'Hexe     Pot azalea       34     'Euratom     Pot azalea       35     'Knut Erwén     Pot azalea       36     'Reinhold Ambrosius     Pot azalea       37     'Eclaireur     Pot azalea       38     'Elsa Kaerger     Pot azalea       39     'Ambrosiana     Pot azalea       40     Leopold-Astrid     Pot azalea       41     Perfe de Swijnaerde     Pot azalea       42     'Tempérance     Pot azalea       43     'Coelestine     Pot azalea       44     Dame Blanche     Pot azalea       45     'Glaser Nr.10     Pot azalea       46     'Madame PB. Van Acker     Pot azalea       47     Tamita     Pot azalea       48     Gloire de Saint Georges     Pot azalea       49     Reinhild     Pot azalea       50     'Teirchillen     Pot azalea       51     '	26	Niobe	Pot azalea	ne riouw soler neuroer vitermann s superou
28     Erolic do Belgique     Pot azalea       29     Pink Dream     Pot azalea       30     "Comtesse de Kerchove     Pot azalea       31     "Paul Schaeme     Pot azalea       32     "Roi Leopold     Pot azalea       33     "Hexe     Pot azalea       34     "Euration     Pot azalea       35     "Kunt Erwén     Pot azalea       36     "Reinhold Ambrosius     Pot azalea       37     "Eclaireur     Pot azalea       38     "Elsa Kaerger     Pot azalea       39     "Ambrosiana     Pot azalea       40     Leopold-Astrid     Pot azalea       41     Perfe de Swijnaerde     Pot azalea       42     "Tempérance     Pot azalea       43     "Coelestine     Pot azalea       44     Dame Blanche     Pot azalea       45     "Glaser Nr. 10     Pot azalea       46     "Madame PB. Van Acker     Pot azalea       47     Tamira     Pot azalea       48     Gloire de Saint Georges     Pot azalea       50     "Erich Danneberg     Pot azalea       51     "Hellmut Vogel     Pot azalea       52     "Mevrouw Marcel Yanbelle     Pot azalea       53     Doctor Heimann	27	*Adventsglocke	Pot azalea	Paul Schaeme×Fritz Sander
29     "Pink Dream"     Pot azalea       30     "Contress de Kerchove     Pot azalea     Wilhelm Scheurer×Deutsche Perle       31     "Paul Schaeme     Pot azalea     Wilhelm Scheurer×Deutsche Perle       32     "Roi Leopold     Pot azalea     Herzog Adolf von Nassau×Amoenum       33     "Hexe     Pot azalea     ApolloXHexe       34     "Euratom     Pot azalea     Paircross 5°       35     "Knut Erwén     Pot azalea     Paircross 4>XPuircross 5°       36     "Reinhold Ambrosius     Pot azalea     Eggebrechtür×Etoile de Noël       37     "Eclaireur     Pot azalea     Eggebrechtür×Etoile de Noël       38     "Isa Kærger     Pot azalea     Eggebrechtür×Etoile de Noël       39     "Ambrosiana     Pot azalea     Pot azalea       40     Leopold-Astrid     Pot azalea     Eggebrechtür×Etoile de Noël       41     Perle de Swijnaerde     Pot azalea     Bud sport Roi Leopold       42     "Tempérance     Pot azalea     Madame PetrickxSpit Fire       43     "Goire de Saint Georges     Pot azalea     Madame PB. Van AckerxPaul Schaeme       44     Dame Blanche     Pot azalea     Madame PB. Van AckerxPaul Schaeme       50     "Erich Danneberg     Pot azalea     Madame PB. Van AckerxPaul Schaeme       5	28	Etoile de Belgique	Pot azalea	
30     'Contresse de Kerchove     Pot azalea       31     'Paul Schaeme     Pot azalea       32     'Roi Leopold     Pot azalea       33     'Hexe     Pot azalea       34     'Euratom     Pot azalea       35     'Knut Erwén     Pot azalea       36     'Reinhold Ambrosius     Pot azalea       37     'Eclaireur     Pot azalea       38     'Elsa Kaerger     Pot azalea       39     'Ambrosiana     Pot azalea       40     Leopold-Astrid     Pot azalea       41     Perle de Swijnaerde     Pot azalea       42     'Tempferace     Pot azalea       43     'Coelestine     Pot azalea       44     Dame Blanche     Pot azalea       45     'Goalest Nr. 10     Pot azalea       46     'Madame P.B. Van Acker     Pot azalea       47     Tamira     Pot azalea       48     Gloire de Saint Georges     Pot azalea       49     Reinhild     Pot azalea       51     'Hellmut Vogel     Pot azalea       54     'Tricdhelm Scherrer     Pot azalea       55     'Mevrouw Andre Heungens     Pot azalea       56     'Rosali     Pot azalea       57     'Merouw Andre Heungens	29	*Pink Dream	Pot azalea	
31       Paul Schaeme       Pot azalea       Wilhelm Scheurer>Deutsche Perle         32       "Roi Leopold       Pot azalea       Herzog Adolf von Nassau×Amoenum         33       "Hexe       Pot azalea       ApolloXHexe         34       "Euratom       Pot azalea       Paircross 5°         35       "Knut Erwén       Pot azalea       Paircross 4>2Paircross 5°         36       "Reinhold Ambrosius       Pot azalea       Hexex?         37       "Eclaireur       Pot azalea       EggebrechtiixEtoile de Noèl         38       "Blas Kærger       Pot azalea       Hexex?         40       Leopold-Astrid       Pot azalea       Hexex?         41       Perle de Swijnaerde       Pot azalea       Hod sport Roi Leopold         42       "Tempérance       Pot azalea       Hod sport Roi Leopold         43       "Coelestine       Pot azalea       Madame Petrick>Spit Fire         44       Dame Blanche       Pot azalea       Madame PB. Van Acker         45       "Glaiser Nr. 10       Pot azalea       Madame PEtrick>Spit Fire         47       Tamira       Pot azalea       Madame PB. Van AckerxPaul Schaeme         50       "Erich Danneberg       Pot azalea       AmbrosianaxPaircross 6°	30	*Comtesse de Kerchove	Pot azalea	
33       'Hoxe       Pot azalea         34       'Euratom       Pot azalea       ApolloxHoxe         34       'Euratom       Pot azalea       ApolloxHoxe         35       'Knut Erwén       Pot azalea       Paircross 4×Paircross 5°         36       'Reinhold Ambrosius       Pot azalea       Hexx </td 37       'Eclaireur       Pot azalea       Eggebrechtik/Etoile de Noèl         38       'Elsa Kaerger       Pot azalea	31	*Paul Schaeme	Pot azalea	Wilhelm Scheurer×Deutsche Perle
33     "Hexe"     Pot azalea     Herzog Adolf von NassauxAmoenum       34     "Euratom     Pot azalea     ApolicxHexe       35     "Knut Erwén     Pot azalea     Paircross 4*xPaircross 5°       36     "Reinhold Ambrosius     Pot azalea     EggebrechtixEtoile de Noèl       37     "Eclaireur     Pot azalea     EggebrechtixEtoile de Noèl       38     "Blsa Kaerger     Pot azalea     EggebrechtixEtoile de Noèl       39     "Ambrosiana     Pot azalea     EggebrechtixEtoile de Noèl       40     Leopold-Astrid     Pot azalea     Herxex?       41     Perfe de Swijnaerde     Pot azalea     Hot azalea       42     "Tempérance     Pot azalea     Bud sport Roi Leopold       43     "Coelestine     Pot azalea     Madame Petrick-Spit Fire       44     Dame Blanche     Pot azalea     Madame Petrick-Spit Fire       45     "Glaser Nr. 10     Pot azalea     Madame Petrick-Spit Fire       46     "Madame PB. Van Acker     Pot azalea     AmbrosianaxErich Danneberg       50     "Erich Danneberg     Pot azalea     Hellmut Vogel Xistral       51     "Hellmut Vogel     Pot azalea     Contesse de KerchovexPaircross 1°       52     "Mevrouw André Heungens     Pot azalea     Madame Petrick-XPink Dream <t< td=""><td>32</td><td>*Roi Leopold</td><td>Pot azalea</td><td></td></t<>	32	*Roi Leopold	Pot azalea	
34     *Euratom     Pot zzalea     ApollóxHexe       35     *Knut Erwén     Pot zzalea     Paircross 4xPaircross 5°       36     *Reinhold Ambrosius     Pot azalea     Hexex?       37     *Eclaircur     Pot azalea     Hexex?       38     *Elas Kaerger     Pot azalea     Hexex?       39     *Ambrosiana     Pot azalea     Hexex?       40     Leopold-Astrid     Pot azalea     Hexex       41     Perle de Swijnaerde     Pot azalea     Hexex       42     "Tempérance     Pot azalea     Bud sport Roi Leopold       43     *Coelestine     Pot azalea     Roi Leopold       44     Dame Blanche     Pot azalea     Madame Petrick>Spit Fire       44     Dame Blanche     Pot azalea     Madame Petrick>Spit Fire       45     *Glaser Nr. 10     Pot azalea     Madame PB. Van Acker>Pot azalea       46     *Madame PB, Van Acker     Pot azalea     Madame PB. Van Acker>Paul Schaeme       51     *Hellmut Vogel     Pot azalea     AmbrosianaxErich Danneberg       52     *Mevrouw Marcel Vanbelle     Pot azalea     Hellmut Vogels/Mistral       53     Doctor Heimann     Pot azalea     Comtese de KerchovexPaircross 1°       54     *Friedhelm Scherrer     Pot azalea     Madame Patrick	33	*Hexe	Pot azalea	Herzog Adolf von Nassau×Amoenum
35     *Knut Erwén     Pot azalea     Paircross 4xPaircross 5°       36     *Reinhold Ambrosius     Pot azalea     Hexex?       37     *Eclaireur     Pot azalea     EggebrechtiixEtoile de Noèl       38     *Elsa Kaerger     Pot azalea     EggebrechtiixEtoile de Noèl       39     *Ambrosiana     Pot azalea       40     Leopold-Astrid     Pot azalea       41     Perfe de Swijnaerde     Pot azalea       42     "Tempérance     Pot azalea       43     *Coelestine     Pot azalea       44     Dame Blanche     Pot azalea       45     "Glaser Nr. 10     Pot azalea       46     *Madame P.B. Van Acker     Pot azalea       47     Tamira     Pot azalea       48     Gloire de Saint Georges     Pot azalea       49     Reinhild     Pot azalea       41     Pot azalea     Madame P.B. Van Acker:       51     "Hellmut Vogel     Pot azalea       52     "Mevrouw Marcel Vanbelle     Pot azalea       53     Doctor Heimann     Pot azalea       54     *Friedhelm Scherrer     Pot azalea       55     *Mevrouw André Heungens     Pot azalea       56     *Rosali     Pot azalea       57     *Kirgfisher     Pot	34	*Euratom	Pot azalea	Apollo×Hexe
36     *Reinhold Ambrosius     Pot azalea     Hexex?       37     *Eclaireur     Pot azalea     Eggebrechtii×Etoile de Noèl       38     *Elsa Kaerger     Pot azalea     Eggebrechtii×Etoile de Noèl       39     *Ambrosiana     Pot azalea     Eggebrechtii×Etoile de Noèl       39     *Ambrosiana     Pot azalea       40     Leopold-Astrid     Pot azalea       41     Perle de Swijnaerde     Pot azalea       42     *Tempérance     Pot azalea       43     *Coelestine     Pot azalea       44     Dame Blanche     Pot azalea       45     *Glaser Nr. 10     Pot azalea       46     *Madame PB. Van Acker     Pot azalea       47     Tamira     Pot azalea       48     Gloire de Saint Georges     Pot azalea       49     Reinhild     Pot azalea       50     *Erich Danneberg     Pot azalea       51     'Hellmut Vogel     Pot azalea       52     'Mevrouw Marcel Vanbelle     Pot azalea       53     Doctor Heimann     Pot azalea       54     *Friedhelm Scherrer     Pot azalea       55     *Mevrouw André Heungens     Pot azalea       56     *Rosali     Pot azalea       57     *Kingfisher     Pot aza	35	*Knut Erwén	Pot azalea	Paircross 4°×Paircross 5°
37     "Eclaireur     Pot azalea     EggebrechtüixEtoile de Noèl       38     "Elsa Kaerger     Pot azalea       39     'Ambrosiana     Pot azalea       40     Leopold-Astrid     Pot azalea       41     Perle de Swijnaerde     Pot azalea       42     "Tempérance     Pot azalea       43     "Coelestine     Pot azalea       44     Dame Blanche     Pot azalea       45     "Glaser Nr. 10     Pot azalea       46     "Madame PB. Van Acker     Pot azalea       47     Tamira     Pot azalea       48     Gloire de Saint Georges     Pot azalea       49     Reinhild     Pot azalea       49     Reinhild     Pot azalea       51     "Hellmut Vogel     Pot azalea       52     "Mevrouw Marcel Vanbelle     Pot azalea       54     "Friedhelm Scherrer     Pot azalea       55     "Mevrouw André Heungens     Pot azalea       56     "Rosal     Pot azalea       57     "Kingfisher     Pot azalea       58     "Flamenco     Pot azalea       59     Ostalett     Pot azalea       60     "Bertina     Pot azalea       61     "Schnee     Pot azalea       62     "Mistr	36	*Reinhold Ambrosius	Pot azalea	Hexe×?
38       "Elsa Kaerger       Pot azalea         39       "Ambrosiana       Pot azalea         40       Leopold-Astrid       Pot azalea         41       Perle de Swijnaerde       Pot azalea         42       "Tempérance       Pot azalea         43       "Coelestine       Pot azalea         44       Dame Blanche       Pot azalea         45       "Glaser Nr. 10       Pot azalea         46       "Madame PB. Van Acker       Pot azalea         47       Tamira       Pot azalea         48       Gloire de Saint Georges       Pot azalea         49       Reinhild       Pot azalea         49       Reinhild       Pot azalea         50       "Erich Danneberg       Pot azalea         51       "Hellmut Vogel       Pot azalea         52       "Mevrouw Marcel Vanbelle       Pot azalea         53       Doctor Heimann       Pot azalea         54       "Friedhelm Scherrer       Pot azalea         55       "Mevrouw André Heungens       Pot azalea         56       "Rosali       Pot azalea         57       "Kingfisher       Pot azalea         58       "Flamenco       Pot azalea <td>37</td> <td>*Eclaireur</td> <td>Pot azalea</td> <td>Eggebrechtii×Etoile de Noèl</td>	37	*Eclaireur	Pot azalea	Eggebrechtii×Etoile de Noèl
39       "Ambrosiana"       Pot azalea         40       Leopold-Astrid       Pot azalea         41       Perle de Swijnaerde       Pot azalea         42       "Tempérance       Pot azalea         43       "Coelestine       Pot azalea         44       Dame Blanche       Pot azalea       bud sport Roi Leopold         45       "Glaser Nr. 10       Pot azalea       Madame Petrick×Spit Fire         46       "Madame P.B. Van Acker       Pot azalea       Madame Petrick×Spit Fire         47       Tamira       Pot azalea       Madame Petrick×Spit Fire         48       Gloire de Saint Georges       Pot azalea       Madame P.B. Van Acker×Paul Schaeme         50       "Erich Danneberg       Pot azalea       Ambrosiana×Erich Danneberg         51       "Hellmut Vogel       Pot azalea       Ambrosiana×Erich Danneberg         52       "Mevrouw André Heungens       Pot azalea       Ambrosiana×Paircoss 6°         53       Doctor Heimann       Pot azalea       Comtesse de Kerchove×Paircoss 1°         54       "Friedhelm Scherrer       Pot azalea       Madame Petrick×Pink Dream         55       "Mevrouw André Heungens       Pot azalea       Comtesse de Kerchove×Paircoss 1°         56       "Rosali	38	*Elsa Kaerger	Pot azalea	
40       Leopold-Astrid       Pot azalea         41       Perle de Swijnaerde       Pot azalea         42       "Tempérance       Pot azalea         43       "Coelestine       Pot azalea         44       Dame Blanche       Pot azalea         45       "Glaser Nr. 10       Pot azalea         46       "Madame P.B. Van Acker       Pot azalea         47       Tamira       Pot azalea         48       Gloire de Saint Georges       Pot azalea         49       Reinhild       Pot azalea         50       "Erich Danneberg       Pot azalea         51       "Hellmut Vogel       Pot azalea         52       "Mevrouw Marcel Vanbelle       Pot azalea         53       Doctor Heimann       Pot azalea         54       "Friedhelm Scherrer       Pot azalea         55       "Mevrouw André Heungens       Pot azalea         56       "Rosali       Pot azalea         57       "Korpiñsher       Pot azalea         58       "Flamenco       Pot azalea         59       Ostalett       Pot azalea         61       "Schnee       Pot azalea         62       "Mistrial       Pot azalea     <	39	*Ambrosiana	Pot azalea	
41       Perle de Swijnaerde       Pot azalea         42       "Tempérance       Pot azalea         43       "Coelestine       Pot azalea         44       Dame Blanche       Pot azalea       bud sport Roi Leopold         44       Dame Blanche       Pot azalea       Roi Leopold×Coelestine         46       "Madame P.B. Van Acker       Pot azalea       Madame Petrick×Spit Fire         47       Tamira       Pot azalea       Madame Petrick×Spit Fire         48       Gloire de Saint Georges       Pot azalea       Madame P.B. Van Acker×Paul Schaeme         50       "Erich Danneberg       Pot azalea       Mabrosiana×Erich Danneberg         51       "Hellmut Vogel       Pot azalea       Hellmut Vogel×Mistral         53       Doctor Heimann       Pot azalea       Comtesse de Kerchove×Paircross 1°         54       "Friedhelm Scherrer       Pot azalea       Madame Petrick×Pink Dream         55       "Mevrouw André Heungens       Pot azalea       Violacea×Reinhold Ambrosius         56       "Rosali       Pot azalea       Violacea×Reinhold Ambrosius         57       "Kingfisher       Pot azalea       Violacea×Reinhold Ambrosius         59       Ostalet       Pot azalea       Memoria Sander×Heiwa-no-hikari	40	Leopold-Astrid	Pot azalea	
42       "Tempérance       Pot azalea         43       "Coelestine       Pot azalea         44       Dame Blanche       Pot azalea       bud sport Roi Leopold         45       "Glaser Nr. 10       Pot azalea       Roi Leopold>Coelestine         46       "Madame P.B. Van Acker       Pot azalea       Madame Petrick×Spit Fire         47       Tamira       Pot azalea       Madame P.B. Van Acker×Paul Schaeme         48       Gloire de Saint Georges       Pot azalea       Madame P.B. Van Acker×Paul Schaeme         50       "Erich Danneberg       Pot azalea       AmbrosianaxErich Danneberg         51       "Hellmut Vogel       Pot azalea       AmbrosianaxErich Danneberg         52       "Mevrouw Marcel Vanbelle       Pot azalea       AmbrosianaxPaircross 6°         53       Doctor Heimann       Pot azalea       Comtesse de Kerchove×Paircross 1°         54       "Friedhelm Scherrer       Pot azalea       Madame Petrick×Pink Dream         55       "Mevrouw André Heungens       Pot azalea       Volacea×Reinhold Ambrosius         56       "Rosali       Pot azalea       Viacea×Reinhold Ambrosius         57       "Kingfisher       Pot azalea       Saka°×Heiwa-no-hikari         60       "Bertina       Pot azalea <td>41</td> <td>Perle de Swijnaerde</td> <td>Pot azalea</td> <td></td>	41	Perle de Swijnaerde	Pot azalea	
43       "Coelestine"       Pot azalea         44       Dame Blanche       Pot azalea       bud sport Roi Leopold×Coelestine         45       "Glaser Nr. 10       Pot azalea       Madame Petrick×Spit Fire         46       "Madame P.B. Van Acker       Pot azalea       Madame Petrick×Spit Fire         47       Tamira       Pot azalea       Madame Petrick×Spit Fire         48       Gloire de Saint Georges       Pot azalea       Madame P.B. Van Acker×Paul Schaeme         50       "Erich Danneberg       Pot azalea       Madame P.B. Van Acker×Paul Schaeme         51       "Hellmut Vogel       Pot azalea       Ambrosiana×Erich Danneberg         52       "Mevrouw Marcel Vanbelle       Pot azalea       Ambrosiana×Paircross 6°         53       Doctor Heimann       Pot azalea       Comtesse de Kerchove×Paircross 1°         54       "Friedhelm Scherrer       Pot azalea       Madame Petrick×Pink Dream         55       "Mevrouw André Heungens       Pot azalea       Violacea×Reinhold Ambrosius         56       "Rosali       Pot azalea       Violacea×Reinhold Ambrosius         57       "Kingfisher       Pot azalea       Hermann Klussman×Zaailing 18         61       "Schnee       Pot azalea       Osaka°×Heiwa-no-hikari <td< td=""><td>42</td><td>*Tempérance</td><td>Pot azalea</td><td></td></td<>	42	*Tempérance	Pot azalea	
44     Dame Blanche     Pot azalea     bud sport Roi Leopold       45     "Glaser Nr. 10     Pot azalea     Roi Leopold×Coelestine       46     "Madame P.B. Van Acker     Pot azalea     Madame Petrick×Spit Fire       47     Tamira     Pot azalea     Madame Petrick×Spit Fire       48     Gloire de Saint Georges     Pot azalea     Madame P.B. Van Acker×Paul Schaeme       50     *Erich Danneberg     Pot azalea     Madame P.B. Van Acker×Paul Schaeme       51     "Hellmut Vogel     Pot azalea     Ambrosiana×Erich Danneberg       52     "Mevrouw Marcel Vanbelle     Pot azalea     Hellmut Vogel×Mistral       53     Doctor Heimann     Pot azalea     Ambrosiana×Erich Danneberg       54     "Friedhelm Scherrer     Pot azalea     Comtesse de Kerchove×Paircross 1°       55     "Mevrouw André Heungens     Pot azalea     Madame Petrick×Pink Dream       57     "Kingfisher     Pot azalea     Violacea×Reinhold Ambrosius       58     "Flamenco     Pot azalea     Violacea×Reinhold Ambrosius       60     "Bertina     Pot azalea     Hermann Klussman×Zaailing 18       61     "Schnee     Pot azalea     Memoria Sander×Heiwa-no-hikari       63     "Lara     Pot azalea     Memoria Sander×Heiwa-no-hikari       64     Aline     Pot aza	43	*Coelestine	Pot azalea	
45       "Glaser N: 10       Pot azalea       Koi Leopold×Coelestine         46       "Madame PB. Van Acker       Pot azalea       Madame Petrick×Spit Fire         47       Tamira       Pot azalea       Madame Petrick×Spit Fire         48       Gloire de Saint Georges       Pot azalea       Madame Petrick×Spit Fire         49       Reinhild       Pot azalea       Madame P.B. Van Acker×Paul Schaeme         50       "Erich Danneberg       Pot azalea       Ambrosiana×Erich Danneberg         51       "Hellmut Vogel       Pot azalea       Hellmut Vogel×Mistral         52       "Mevrouw Marcel Vanbelle       Pot azalea       Ambrosiana×Paircross 6°         53       Doctor Heimann       Pot azalea       Contesse de Kerchove×Paircross 1°         54       "Friedhelm Scherrer       Pot azalea       Madame Petrick×Pink Dream         55       "Mevrouw André Heungens       Pot azalea       Violacea×Reinhold Ambrosius         56       "Rosali       Pot azalea       Violacea×Reinhold Ambrosius         57       "Kingfisher       Pot azalea       Violacea×Reinhold Ambrosius         58       "Flamenco       Pot azalea       Meadame Petrick×Pink Dream         61       "Schnee       Pot azalea       Meadame Petrick×Pink Dream      <	44	Dame Blanche	Pot azalea	bud sport Roi Leopold
46       Madame Petrick×Spit Fire         47       Tamira       Pot azalea         48       Gloire de Saint Georges       Pot azalea         49       Reinhild       Pot azalea         50       "Erich Danneberg       Pot azalea         51       "Hellmut Vogel       Pot azalea         52       "Mevrouw Marcel Vanbelle       Pot azalea         53       Doctor Heimann       Pot azalea         54       "Friedhelm Scherrer       Pot azalea         55       "Mevrouw André Heungens       Pot azalea         56       "Rosali       Pot azalea         57       "Kingfisher       Pot azalea         58       "Flamenco       Pot azalea         59       Ostalett       Pot azalea         60       "Bertina       Pot azalea         61       "Schnee       Pot azalea         62       "Mistral       Pot azalea         63       "Lara       Pot azalea         64       Aline       Pot azalea         65       Kosmos       Pot azalea         66       Dorothy Gish       Pot azalea         67       Heidi       Pot azalea         68       "James Belton	45	*Glaser Nr. 10	Pot azalea	Roi Leopold×Coelestine
47       Iamira       Pot azalea         48       Gloire de Saint Georges       Pot azalea         49       Reinhild       Pot azalea         50       "Erich Danneberg       Pot azalea         51       "Hellmut Vogel       Pot azalea         52       "Mevrouw Marcel Vanbelle       Pot azalea         53       Doctor Heimann       Pot azalea         54       "Friedhelm Scherrer       Pot azalea         55       "Mevrouw André Heungens       Pot azalea         56       "Rosali       Pot azalea         57       *Kingfisher       Pot azalea         58       *Flamenco       Pot azalea         59       Ostalett       Pot azalea         60       "Bertina       Pot azalea         61       *Schnee       Pot azalea         62       "Mistral       Pot azalea         63       *Lara       Pot azalea         64       Aline       Pot azalea         65       Kosmos       Pot azalea         66       Dorothy Gish       Pot azalea         67       Heidi       Pot azalea         68       *James Belton       Pot azalea         69       "Schuman	46	Madame P.B. Van Acker	Pot azalea	Madame Petrick×Spit Fire
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**Table 1** Variety name, type of hybrid and parentage of the genotypes analysed with AFLP. The 34 plants included in the kinship analysis are preceded by a asterisk (\*)

Number <sup>a</sup>	Variety	Туре	Parentage <sup>b, c</sup>	
13	R. nakaharai	Species		
18	R. simsii	Species		
19	R. tashiroi	Species		
73	R. indicum	Species		
10	R. kiusianum	Species		
14	R. noriakianum	Species		
16	R. mucronatum	Species		
17	R. scabrum	Species		
72	R. scabrum	Species		

<sup>a</sup> Numbers refer to the labels of Fig. 3

<sup>b</sup> Parents indicated with ? are unknown; if no pedigree data are given, both parents are unknown

<sup>c</sup> Pedigree information for plants not analysed with AFLP but included in the kinship analysis: Herrmann's Superba ('Madame P.B. Van Acker'×'Paul Schaeme'); Mevrouw Jozef Heursel ('Vio-

molecular marker technique for assessing genetic conformity for both the study and conservation of natural diversity and for the characterisation of breeding gene pools, e.g. in soybean (Maughan et al. 1996), lettuce (Hill et al. 1996), tea (Paul et al. 1997), sunflower (Hongtrakul et al. 1997) and maize (Ajmone Marsan et al. 1998).

Fluorescent fragment labelling applicable on automated sequencers can add an extra advantage by providing a radioactive-free system. Moreover, if a combination of fluorescent dyes with distinct emission spectra can be multiplexed in one lane, higher throughput per gel and the application of an internal size standard in each lane can be achieved. Such systems can enable automated size determination and scoring of the fragments, resulting in a substantial time-saving mainly due to the reduced post-electrophoresis steps for band scoring. This gain is most evident when one has to score a high number of polymorphic fragments in a large set of lowly related plants.

Due to the large number of markers AFLP might yield, one needs consistent criteria to select the more informative markers. The main objective of the study presented here was to validate the reliability of well-defined marker selection parameters to handle AFLP data output. Instead of selecting a subset of polymorphic bands with the eye, we applied thresholds for marker signal intensity and marker frequencies over the data set. Although their definition was facilitated by fluorescent AFLP on a DNA sequencer, these parameters can be readily applied to any band scoring system capable of quantifying band signal intensity.

A first validation focussed on the sensitivity of the resulting ordination for different marker selection parameter settings: discarding weak bands or imposing the degree of polymorphism of the markers included. Two different resemblance measures, the Simple Matching and the Jaccard coefficient, were evaluated as both represent a different group of measures for binary data: the first being a symmetrical measure rewarding the double absence of a marker in a pair of plants as a fact contributlacea'×'Hexe'); Osaka ('Madame Petrick'×'Pink Dream'); Paircross 1 ('Reinhold Ambrosius'×'Madame Petrick'); Paircross 2 ('Osaka'×'Pink Dream'); Paircross 3 ('Osaka'×'Pink Dream'); Paircross 4 ('Hermann Seidel'×'Professor Wolters'); Paircross 5 ('Paul Schaeme'×'Apollo'; Paircross 6 ('Coelestine'×?)

ing to similarity, the second an asymmetrical measure neglecting double absences. A second validation, although less statistically quantifiable, could be obtained from the genetic diversity study itself, examining a well characterised breeders' collection of 75 genotypes of Belgian pot azalea (*Rhododendron simsii* hybrids) and other related evergreen azaleas belonging to the *Tsutsusi* subgenus (Chamberlain and Rae 1990). The genetic conformity revealed by AFLP was compared with the known pedigree (34 genotypes), the geographical origin or the supposed relationship based on the morphology of the genotypes studied.

## **Materials and methods**

#### Plant material

In total 75 azalea genotypes were analysed (Table 1). They were chosen from three distinguishable subgroups within the evergreen azaleas: pot azaleas (*Rhododendron simsii* hybrids; 55 genotypes), Hirado azaleas (*R. scabrum* hybrids; 4 genotypes) and Kurume azaleas (hybrids of *R. kiusianum* var 'kiusianum' and var 'sataense' and *R. kaempferi*; 7 genotypes). Apart from *R. simsii*, at least three other species *R. indicum*, *R. mucronatum* and *R. scabrum* are accepted to be the ancestors of pot azaleas (Galle 1987). Therefore, also included. Within the pot azaleas, some varieties obtained from bud sporting were included (two 'Nordlicht' type of bud sports from 'Hellmut Vogel', one altered in flower colour, the other also in growth behaviour; 'Dame Blanche' bud sport from 'Roi Leopold' and 'Heidi' bud sport from 'Rosali').

#### DNA isolation

Young leaf material (approximately 1 g fresh weight) was harvested from 2-year-old potted plants maintained in the greenhouse. At harvest plant material was immediately immersed in liquid nitrogen and subsequently lyophilised for 48 h. The dry material was vacuum-packed for storage at  $-20^{\circ}$ C until DNA extraction. Stored material was ground using a Culatti mechanical mill. Different DNA isolation protocols based on Dellaporte et al. (1983), Doyle and Doyle (1987), Greenwood et al. (1989), Kobayashi et al. (1995) and Weising et al. (1991) were tested with the final result being the following procedure. To 25–75 mg lyophilised ground tissue, 10 ml of a 50 mM TRIS-HCl (pH 8) containing 5 mM

EDTA, 350 mM sorbitol, 0.1% β-mercaptoethanol, 10% PEG (MW 8000) and 0.001% BSA were added. This mixture was homogenised for 30 s by inverting the tubes and then filtered through Miracloth (Calbiochem) to remove coarse cell debris. The nuclei fraction was pelleted by centrifugation (2500 g, 5 min,  $4^{\circ}$ C). To the nuclei fraction, 1 ml CTAB extraction buffer (100 mM Tris-HCl pH 8, containing 2% CTAB, 20 mM EDTA, 1.4 M NaCl, 0.5 mM Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, 0.4% β-mercaptoethanol and 1% PVP MW 40000) and RNase (10 U) were added. Samples were incubated for 40 min. at 65°C. Afterwards, samples were homogenised with 1 ml chloroform/isoamylalcohol (24/1) and centrifuged for 15 min. at 10 000 g. The supernatant was transferred to a fresh tube and the DNA precipitated with 1 ml of ice cold (-20°C) isopropanol. After centrifugation (5000 g; 15 min), the pellet was washed with EtOH (76%) - 0.2 M NaOAc, dried and dissolved in water. DNA concentration and quality were constantly checked relative to a standard series of lambda-DNA after electrophoresis on a 1.5% TAE buffered agarose gel.

#### AFLP reactions and PAGE

AFLP was performed using the commercially available kit from Perkin-Elmer Biosystems for fluorescent fragment detection (Perkin-Elmer 1995). *Eco*RI and *Mse*I were used for DNA digestion. Adapter ligation, preselective and selective amplification were as in the manufacturers' protocols. Selective amplification was done using fluorescent-labelled EcoRI-MseI primer combinations with 6 selective bases. The primer combinations used were: *Eco*RI-ACT/*Mse*I-CTA (PC1), *Eco*RI-ACT/*Mse*I-CAT (PC2), *Eco*RI-AAG/*Mse*I-CTA (PC3). PCR amplifications were performed using a Perkin-Elmer 9600. AFLP fragments were separated by PAGE on a ABI Prism 377 DNA Sequencer on 36cm gels using 4.25% denaturing polyacrylamide (4.25% acrylamide/bisacrylamide 19/1, 6 *M* urea in 1×TBE). GS-500-ROX labelled size standard (Perkin Elmer) was loaded on each lane in order to facilitate the automatic analysis of the gel and the sizing of the fragments.

#### Band scoring and statistical analyses

During a run on the ABI 377, the fluorescent signal in each lane is being recorded continuously. For each fluorescent dye electropherograms are extracted that are comparable to the densitometric curves obtained after scanning of an autoradiogram. GENESCAN 2.1 was used to estimate detection time, signal peak height and surface for each fragment. Sizing of the fragments was performed by the Genescan software module by interpolation to the internal lane standard according to the Local Southern algorithm. Only the fragments between 70 bp and 450 bp were used for scoring. Resolution of the gel system (i.e. the capacity to separate 2 subsequent bands in one lane) is 1 bp. However, due to lane-to-lane variation and differences in the interpolation of the standard the scoring of the same band position between different lanes varied within 1 bp. After export of the Genescan data to Microsoft Access, these variations in fragment size were assigned to the corresponding categories and a scoring table (1/0) was generated. Using Access queries, we set different filters towards signal peak height and marker frequency. Calculation of similarity coefficients, construction of dendrograms (UPGMA), Mantel analysis and principal co-ordinates analysis were performed by the modules SIMIL, CLUSTER, MANTEL and PCOORD of the "R package" (Legendre and Vaudor 1991). Mantel analysis computed standardised Mantel's statistics between two resemblance matrices. The significance of the statistic was evaluated by permutations (1000×) and expressed as a probability (Smouse et al. 1986). Pedigree analysis was performed by defining an "unrelated" ancestor population first, i.e. by tracing back the pedigree until all parents were unknown. For all plants in the pedigree, kinship coefficients (r) were calculated using KIN (Tinker and Mather 1993) and were turned into a distance (1-r).

## **Results and discussion**

# Effectiveness of the primer combinations for *R. simsii* hybrids

For azalea, only primer combinations with 6 selective bases that had been tested previously were used (De Riek et al. 1997). They generate approximately 70-90 AFLP fragments per reaction. Intense bands, indicative of repetitive fragments, were not observed in the sets used. PC1 generated 219 unique fragments when all plant reactions were scored; PC2, 198 and PC3, 231. A visual check on the electropherograms did not show any polymorphisms between the initial variety 'Hellmut Vogel' and its two bud sports, or between 'Heidi' and 'Rosali' or 'Dame Blanche' and 'Roi Leopold'. From a more extensive study, similar results were obtained using several PC and different bud sports of 'Hellmut Vogel' (De Riek et al. 1997). The parentage of plants with known genitors was confirmed on a visual check. No bands were present in the offspring that could not be traced back in the putative parents.

Sensitivity of the ordination for different marker selection parameter settings

Scoring AFLP gels can be an error-prone task due to the fact that in larger sets of unrelated plants large ranges of fragments differing only 1 bp from each other might occur. Also, there might be a tendency to discard weak, infrequent and/or constant bands. When discarding constant bands, for example, a quite obvious effect is possible: pairwise similarities between plants will decrease. However, whether the plant order in classifications will be changed is mostly unclear. Another uncertainty to deal with is the impact of a change in the quality of AFLP gels (variations in radioactive or fluorescent signal, different dyes or reactions) on the final results. When carrying out automated scoring, these kind of risks might be estimated as being higher because of the lack of a check by eye. Therefore, consistent marker selection criteria are required.

Selection for the average fluorescent signal intensity of a marker was performed using three thresholds: 50, 120 and 300 units. Before marker selection, all data were collected under a Genescan baseline setting of 50 units (as recommended by the manufacturer) and attributed to a marker category. The average signal intensity of all bands scored as the same marker was then used as a criterion. If the Genescan baseline setting is raised beforehand, it is likely to discard bands that are present but have accidentally a low signal. This is mainly due to the fact that during extraction of the electropherogram only part of the total band signal (three data channels adjacent to a lane tracking line) is being recorded by the software. Peak height and surface can thus be influenced by any shift of the tracking line from the centre of a band.



**Fig. 1A, B** AFLP marker distributions based on: **A** the frequency they appeared in the set of genotypes analysed and **B** their average fluorescent signal intensity in the gels. Different marker selection thresholds are indicated

Selection to marker frequency was performed in two ways: (1) exclusion of all rare markers (f<0.15) and (2) exclusion of all rare and abundant markers (0.15 < f<0.85). All fingerprinted plants were taken into account for the frequency calculation. The effect of these thresholds can be deduced from the marker distributions as shown in Fig. 1; Table 2 summarises the number of markers included in the analysis for each of the nine cases. The number of markers decreases drastically when frequency thresholds are applied. Merely rare markers are lost. Without selection to signal intensity, only 50 markers appeared to be abundant. This value was approximately the same under higher signal intensity thresholds (48 abundant markers at 120 units; 42 at 300 units). Therefore, when applying signal intensity selection, one is enriching for abundant markers. The question if abundant markers were also the more homozygous ones could not be evaluated because the Genescan data extraction mode as used (as mentioned before) did not allow the reproducible co-dominant scoring of AFLP fragments based on peak height or surface.

Each of the nine parameter settings (Table 2) was used as an input for the calculation of pair-wise similarity matrices. The use of the Simple Matching (symmetrical) and the Jaccard coefficient (asymmetrical measure) 1159

Marker frequency selection	Selection for average fluorescent signal intensity (units)			
	>50	>120	>300	
None f>0.15	648 245	389 179	166 102	
0.15 <f<0.85< td=""><td>195</td><td>131</td><td>60</td></f<0.85<>	195	131	60	

were evaluated. For AFLPs, different alleles in general cannot be distinguished as separate bands, and there might be some distrust in using a symmetrical coefficient. The absence of an AFLP marker in 2 plants can be caused by completely different genetic mutations and should not be considered equally. On the other hand, until it has been checked by segregation in mapping populations or sequencing of the fragments, one is not completely sure that a band present at the same gel position is identical.

To evaluate the sensitivity of the final ordination for different marker selection parameter settings, we used two approaches: (1) detection of differences on average and range of the similarity matrices, and (2) statistical tests (Mantel test) on the correlation between pairs of matrices (the construction of dendrograms for all cases and the detection of differences in these final ordinations were not considered to be feasible using 75 plants and 18 matrices). Since the results could have been altered by the reduction in the number of data sets when applying different marker selection criteria, the effect of a random reduction of the data set was evaluated first.

By resampling randomly an equal number of markers as for the eight restricted cases, five data sets per case were created. In general, averages and ranges of the similarity matrices for the resampled data sets were close to the values of the initial data set with 648 markers (results not shown). Using the Simple Matching coefficient on the initial data set, the average similarity was 0.82, ranging from a minimum of 0.69 to a maximum of 0.94 (Table 3). The average similarity for all resampled similarity matrices varied between 0.810 and 0.825, a very close range around the value for the initial data set. The minimum similarity varied between 0.55 (resampling of 60 markers) and 0.68 (resampling of 389 markers). The maximum similarity was between 0.95 and 0.98. For the Jaccard coefficient on the initial data set, the average similarity was 0.45, ranging from a minimum of 0.21 to a maximum of 0.78 (Table 3). The averages of all sampled similarity matrices were from 0.43 to 0.47; the minimum from 0.04 to 0.18 and the maximum from 0.77 (resampling of 389 markers) to 0.94 (resampling of 60 markers). In conclusion, if deviations were observed, it was for the minimum and maximum similarities, but only for very reduced data sets (60 markers). Therefore, a minor effect had to be attributed to the reduction of the initial data set when evaluating different marker selection settings.

Table 3 Average, minimum and maximum values of the pairwise similarity matrices based on Simple Matching and Jaccard coefficients, derived from AFLP data using different settings for selection for marker frequency and average marker signal intensity

Simple Matching coefficient					
Marker frequency selection	Selection for average fluorescent signal intensity (units)				
	>50	>120	>300		
None f>0.15 0.15 <f<0.85< td=""><td>0.82 (0.69–0.94) 0.66 (0.50–0.92) 0.59 (0.43–0.90)</td><td>0.81 (0.63–1) 0.69 (0.50–1) 0.58 (0.38–1)</td><td>0.81 (0.60–1) 0.74 (0.47–1) 0.60 (0.35–1)</td></f<0.85<>	0.82 (0.69–0.94) 0.66 (0.50–0.92) 0.59 (0.43–0.90)	0.81 (0.63–1) 0.69 (0.50–1) 0.58 (0.38–1)	0.81 (0.60–1) 0.74 (0.47–1) 0.60 (0.35–1)		
Jaccard Coefficient					
Marker frequency selection	Selection for average fluorescent signal intensity (units)				
	>50	>120	>300		
None f >0.15 0.15 <f <0.85<="" td=""><td>0.45 (0.21–0.78) 0.53 (0.28–0.87) 0.36 (0.14–0.81)</td><td>0.53 (0.26–0.97) 0.60 (0.34–1) 0.39 (0.16–1)</td><td>0.65 (0.32–1) 0.68 (0.29–1) 0.45 (0.14–1)</td></f>	0.45 (0.21–0.78) 0.53 (0.28–0.87) 0.36 (0.14–0.81)	0.53 (0.26–0.97) 0.60 (0.34–1) 0.39 (0.16–1)	0.65 (0.32–1) 0.68 (0.29–1) 0.45 (0.14–1)		

The average value of a similarity matrix and ranges (Table 3) can provide different categories of information: (1) the general similarity in the set of plants, (2) the discrimination capacity between genotypes and (3) the robustness of the ordination to genotyping or scoring variability. General similarity for the set of plants was highly influenced by different marker selection parameter settings (Table 3). Exclusion of both rare and abundant markers provoked a decrease in the average values for both coefficients. Ordinations based on the Simple Matching coefficient were more affected as expected from the nature of this coefficient. For Jaccard, exclusion of rare markers induced a slight increase of the general similarity; the opposite was observed for the Simple Matching coefficient. Selection for more intensive markers had little influence or provoked an increase, depending on the setting for selection for marker frequency and the coefficient used.

Regarding the discrimination capacity between genotypes, the generally broader ranges of similarity obtained in Jaccard-based pairwise comparisons allowed better separation of individual genotypes. For both coefficients, imposing any restriction to the markers included in the analysis improved the discriminatory power of the ordination (as evaluated by the ranges). Only little influence was seen on the range by increasing the average fluorescent signal intensity from 120 to 300. The best discrimination capacity was obtained when both rare and abundant markers were excluded from the analysis.

The robustness of the ordination to genotyping or scoring variability could be evaluated because genetically nearly identical genotypes (bud sports) were included that showed no polymorphisms on the electropherograms. For certain settings, the maximum similarity was below 1, and bud sports were wrongly separated as distinct. Two facts can be the cause: (1) artefact bands from imprecise band definition by the Genescan software and (2) variations in signal intensities leading to the underscoring of bands with a peak height around the Genescan detection setting (50 units). Eliminating rare bands, excluding most of the artefact bands, gave no or only slight improvement; raising the average fluorescent signal intensity above the detection setting was much more effective.

As a test for the correlation of the enclosed data configuration, a standardised Mantel's statistic was calculated on pairs of similarity matrices. Two types of tests were aimed for. Firstly, using a Jaccard or Simple Matching coefficient, what is the sensitivity of an ordination for the variation in frequency selection under constant signal intensity settings (Table 4: upper panels) or, vice versa, for a variation in signal intensity selection under constant frequency selection settings (Table 4: lower panels)? Secondly, under the same marker selection settings, what is the correlation between an ordination based on the Jaccard versus the Simple Matching coefficient (Table 5)? The first assay can be considered as a test for the reliability of the classification to improper settings of marker selection criteria; the second as a test for the independence of the classification towards the use of a certain similarity measure. Using the Jaccard coefficient, the correlation between the similarity matrices was not affected a lot by the exclusion of the rare and abundant markers (Table 4, upper panels, values below the diagonal). For the Simple Matching coefficient (values above the diagonal), however, correlation decreased strongly. This effect was stronger when no signal intensity selection was applied. Marker selection to average fluorescent signal intensity did not reveal deviant behaviour between the use of Jaccard and Simple matching similarity (Table 4, lower panels). Correlation decreased when only the more intensive bands were scored. This effect was stronger if the rare and abundant markers were excluded. Correlation between Jaccard and Simple matching similarity matrices (Table 5) decreased when rare and abundant markers were excluded. Marker selection towards signal intensity increased this correlation. This effect was most clear when no frequency selection was applied.

**Table 4** Standardised Mantel's statistic<sup>a</sup> between similarity matrices derived from AFLP data using different settings for selection for marker frequency and average marker signal intensity

Variation in frequency selection under constant average fluorescent signal intensity selection (above diagonal: using the Simple Matching coefficient; below diagonal: Jaccard coefficient)

Average signal intensity	Marker frequency selection	None	f>0.15	0.15 <f<0.85< th=""></f<0.85<>
>50	None f>0.15 0.15 <f<0.85< td=""><td>0.95 0.90</td><td>0.85 - 0.96</td><td>0.75 0.96 </td></f<0.85<>	0.95 0.90	0.85 - 0.96	0.75 0.96 
>120	None f>0.15 0.15 <f<0.85< td=""><td> 0.96 0.90</td><td>0.90 - 0.95</td><td>0.77 0.94 -</td></f<0.85<>	 0.96 0.90	0.90 - 0.95	0.77 0.94 -
>300	None f>0.15 0.15 <f<0.85< td=""><td>0.98 0.87</td><td>0.95 - 0.91</td><td>0.81 0.90 -</td></f<0.85<>	0.98 0.87	0.95 - 0.91	0.81 0.90 -

Variation in average fluorescent signal intensity selection under constant frequency selection (above diagonal: using the Simple Matching coefficient; below diagonal: Jaccard coefficient)

Marker frequency selection	Average signal intensity	>50	>120	>300
None	>50 >120 >300	0.97 0.89	0.95 - 0.93	0.85 0.92
f>0.15	>50 >120 >300	0.96 0.88	0.9 - 0.91	0.80 0.88 -
0.15 <f<0.85< td=""><td>&gt;50 &gt;120 &gt;300</td><td>0.93 0.83</td><td>0.80 - 0.86</td><td>0.72 0.81 -</td></f<0.85<>	>50 >120 >300	0.93 0.83	0.80 - 0.86	0.72 0.81 -

<sup>a</sup> Probabilities (*P*) evaluated by 1000 permutations were always equal to 0.001

## Correlation between similarities based on molecular markers and kinship

To compare classifications using molecular data to the known or accepted relationship of the genotypes studied, we compared the Jaccard and Simple Matching based similarity matrices obtained according to the marker selection criteria mentioned above to a pedigree-based distance matrix by Mantel analysis. Pedigree analysis was performed for 34 cultivars (Table 1) by defining an "unrelated" ancestor population for which no further pedigree information was available. For most of the cultivars, only three to maximally five generations could be traced back. As can be supposed from the history of R. simsii hybrids, many of these "unrelated" ancestors might in fact be highly related. Genetic similarity by descent was evaluated using kinship coefficients. Kinship has been defined as the probability that alleles of a given locus are identical by descent. It can also be considered as an estimate for the degree of genetic similarity between two individuals (Malécot 1948). For all pairs of plants in the pedigree (in total 75), pairwise kinship coefficients were calculated using KIN (Tinker and Mather 1993) and turned into a distance (1-r). To yield a symmetrical  $(34 \times 34)$  distance matrix as an overall estimate of the genetic distance based on pedigree, we calculated Euclidean distances on the  $34 \times 75$  partial matrix. Results are also presented as a dendrogram (Fig. 2). Starting from the AFLP data sets for 75 plants, under the differ-

 Table 5
 Standardised Mantel's statistic between a Simple Matching coefficient based and a Jaccard coefficient-based similarity matrix, derived from AFLP data under identical settings for selection for marker frequency and average marker signal intensity

Average signal intensity	Marker frequency selection	Standardised Mantel's statistic <sup>a</sup>
>50	None f>0.15 0.15 <f<0.85< td=""><td>0.96 0.92 0.81</td></f<0.85<>	0.96 0.92 0.81
>120	None f>0.15 0.15 <f<0.85< td=""><td>0.98 0.97 0.90</td></f<0.85<>	0.98 0.97 0.90
>300	None f>0.15 0.15 <f<0.85< td=""><td>0.99 0.98 0.89</td></f<0.85<>	0.99 0.98 0.89

<sup>a</sup> Probabilities (*P*) evaluated by 1000 permutations were always equal to 0.001

ent marker selection conditions,  $34 \times 34$  similarity matrices using Jaccard and Simple Matching similarities were extracted. A standardised Mantel's statistic was calculated on each pair of similarity matrices and the pedigree-based distance matrix (Table 6). Values for the statistic were, in general, low, however probabilities evaluated by permutation were significant for several marker selection parameter settings. The most significant correlations were obtained using the Simple Matching coefficient with no or moderate selection to signal intensity and ex-

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**Fig. 2A, B** Ordination of azalea cultivars, based on: **A** pairwise kinship coefficients calculated on the pedigree (Euclidean distance; UPGMA-clustering) and **B** AFLP data (marker selection settings: 0.15<f<0.85, I>120; Simple Matching coefficient; UPGMA clustering). Corresponding clusters are indicated



Table 6 Standardised Mantel's statistic (with indication of the probabilities) between similarity matrices based on Simple Matching and Jaccard coefficients, using different settings for selection for marker frequency and average marker signal intensity, and an Euclidean Distance matrix derived from pairwise kinship coefficients calculated on the pedigree

Simple Matching coefficient				
Marker frequency selection	Selection for average fluorescent signal intensity (units)			
	>50	>120	>300	
None f>0.15 0.15 <f<0.85< td=""><td>0.094 (P: 0.132) 0.219 (P: 0.006) 0.236 (P: 0.002)</td><td>0.117 (<i>P</i>: 0.108) 0.220 (<i>P</i>: 0.010) 0.235 (<i>P</i>: 0.132)</td><td>0.158 (<i>P</i>: 0.078) 0.181 (<i>P</i>: 0.051) 0.199 (<i>P</i>: 0.015)</td></f<0.85<>	0.094 (P: 0.132) 0.219 (P: 0.006) 0.236 (P: 0.002)	0.117 ( <i>P</i> : 0.108) 0.220 ( <i>P</i> : 0.010) 0.235 ( <i>P</i> : 0.132)	0.158 ( <i>P</i> : 0.078) 0.181 ( <i>P</i> : 0.051) 0.199 ( <i>P</i> : 0.015)	
Jaccard Coefficient				
Marker frequency selection	Selection for avera	Selection for average fluorescent signal intensity (units)		
	>50	>120	>300	
None f>0.15 0.15 <f<0.85< td=""><td>0.149 (<i>P</i>: 0.068) 0.212 (<i>P</i>: 0.009) 0.227 (<i>P</i>: 0.002)</td><td>0.143 (P: 0.082) 0.194 (P: 0.023) 0.193 (P: 0.016)</td><td>0.155 (<i>P</i>: 0.075) 0.163 (<i>P</i>: 0.075) 0.158 (<i>P</i>: 0.038)</td></f<0.85<>	0.149 ( <i>P</i> : 0.068) 0.212 ( <i>P</i> : 0.009) 0.227 ( <i>P</i> : 0.002)	0.143 (P: 0.082) 0.194 (P: 0.023) 0.193 (P: 0.016)	0.155 ( <i>P</i> : 0.075) 0.163 ( <i>P</i> : 0.075) 0.158 ( <i>P</i> : 0.038)	

cluding rare and abundant markers (Mantel's statistic of 0.23; probability of 0.002). The corresponding dendrogram is presented in Fig. 2. Jaccard-based similarity matrices tended to have a lower correlation with pedigree than those based on the Simple Matching coefficient. Exclusion of rare markers always resulted in a better correlation between similarities based on molecular data and pedigree. This effect was predominant under no or moderate signal intensity selection. If only intensive markers were selected, there was a tendency to a lower correlation with pedigree-based similarity. Analysis of Fig. 2 indicates that for 10 pairs or groups, a high similarity expected from pedigree is affirmed by AFLP analysis. However, the structure in the AFLP-based ordination at a lower level of similarity did not correspond to the pedigree based ordination. Evaluating pedigrees as described resulted in plants being mainly grouped as a function of common ancestors. This was biased by some incomplete pedigrees, preferentially grouping descendants to the most important ancestor. For example, 'Hellmut Vogel' is closely clustered with 'Erich Danneberg', one of its parents. 'Erich Danneberg' in its turn is a direct descendant of 'Madame Pierre B. Van Acker' and 'Paul Schaeme' in the same cluster. However, 'Ambrosiana', the other parent of 'Hellmut Vogel', is separately clustered to its daughter 'Friedhelm Scherrer' and its granddaughter 'Otto'. This might be the reason for the low Mantel's statistics between pedigree and AFLPbased ordinations. The limited number of generations in the pedigrees caused an overestimation of the distances between groups where pedigree information for common ancestors is missing but where they may be highly related. The AFLP analysis grouped varieties with similar morphological characters better. Some examples are as follows. 'Ambrosiana', 'Otto', 'Reinhold Ambrosius', 'Friedhelm Scherrer' and 'Adventsglocke' are cultivars with similar growth habit, dark green elliptic leaves, mid season flowering and carmine red double flowers. In the pedigree based analysis, 'Pink Dream' is most closely clustered to 'Schuman', an inbred between two half-sibs

of 'Pink Dream'. But phenotypically it shares light-green leaves, single light-pink-coloured flowers and a lateflowering time with its daughter 'Rosali'. In the AFLPbased analysis, Hirado azaleas and close relatives 'Heiwa-no-hikari', 'Lara', 'Mistral' and 'Mevrouw Marcel Vanbelle' ('Mistral'×'Hellmut Vogel'), which share growth habit, fast growth and fragrant flowers, are grouped better.

#### Classification of the breeders' gene pool

Final classification of the breeders' gene pool was performed using a data set with 179 markers (Table 1; f>0.15; average fluorescent signal intensity: 120 units). The choice of this particular set was based on the validation study performed: a fairly large data set with moderate exclusion of markers with a lower intensity, no exclusion of abundant markers, which resulted in a good correlation with pedigree. However, as has been previously discussed, other settings are possible that correlate to the results obtained by this particular one. Using this data set classification in dendrograms appeared to be independent of the use of Jaccard or Simple Matching coefficient or Euclidean distance (results not shown). Principal co-ordinate analysis was used to produce a two-dimensional ordination for the breeders' gene pool (Fig. 3). The current pot azalea assortment was clearly separated from the wild *Rhododendron* species. The latter clustered together with the Kurume azaleas. Hirado azaleas were separately grouped together with R. scabrum. Both Kurume and Hirado azaleas were grouped with their most important ancestor. Within the R. simsii hybrids two subgroups may be distinguished. The first group contains the archetype of a Belgian pot azalea – globular shape, dark-green leaves, early flowering and carmine red double flowers – although it has never been created so far in all its details as a single genotype. It is best characterised by members as 'Madame Petrick' (1880), 'Paul Schaeme' (1890), 'Ambrosiana' (1948) and 'Rein**Fig. 3** PCO ordination for the breeders' collection of evergreen azalea, based on an AFLP data set with 179 markers (marker selection settings: f>0.15, I>120; Simple Matching coefficient). See Table 1 for variety names of ID-numbers



hold Ambrosius' (1930), which before 'Hellmut Vogel' (1967) were the most cultivated commercial pot azaleas. The second subgroup is a more loose group of cultivars, intermediate to the other groups. They can be generally typified as flowering late and having single or half-double flowers. A lot of them are older cultivars, e.g. 'Coelestine', 'Professor Wolters', 'Leopold-Astrid' or 'Tempérance'. Some are cultivars that originate from intermediate crosses: 'Cheops', a new pyramidal azalea created from R. noriakianum, 'Lara' and 'Mistral', crosses between Hirado azaleas and R. simsii hybrids; 'Directeur Van Slycken', a cross between Kurume azalea and a R. simsii hybrid. 'Coelestine', a very old cultivar of unknown origin, is also probably an intermediate form. It shares its globular shape, single flowers and late-flowering date with *R. kiusianum* and the Kurume azaleas. Part of this character is also present in its daughter 'Glaser Nr. 10'. 'Dorothy Gish', 'Kingfisher', 'Hexe' and 'Euratom' show a hose-in-hose type of flower, i.e. a flower of which the calyx is enlarged and coloured as a second corolla. This trait is typical for Kurume azaleas as 'Kirin' and 'Rex'. It is believed to be a mutation. The habit of 'Tempérance' and 'James Belton' is very atypical for R. simsii hybrids: both are late-flowering, very woody and erect: 'Tempérance' shows very uncommon lilac to blue flowers, 'James Belton' has tiny, green, hairy leaves.

## Conclusions

In this relatively large azalea gene pool study fluorescent AFLP resulted in a primary data set with a high number of fragments. The selection of markers to be included in ordination analysis was performed by imposing filter thresholds to marker frequency and signal intensity. Validation of marker selection parameters showed that a range of settings can be found that give ordinations that are highly correlated to each other, that are merely independent of the use of Jaccard or Simple Matching coefficient and that show good agreement with known plant relationships based on pedigree or cultivar origin. Although it is demanding because of the technical infrastructure (DNA sequencer and computer equipment), fluorescent detection of AFLPs facilitates the generation of sufficient numbers of polymorphic markers. The number of PCs to be tested in a gene pool study can be restricted because automated fragment scoring can supply the capacity to register each fragment arising from a single AFLP primer combination. The use of consistent marker selection criteria like marker frequency and fluorescent signal intensity can then be of great help.

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