Specific alleles as individual molecular markers and its association for sustainable breeding program in coconut palm

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Abstract. Molecular markers through individual identification have been used to protect plant varieties, identify varieties, purity test of seeds, and parented analysis in palm oil breeding programs. However, such identification has not been intensively developed on coconut palm. Therefore, this study aimed to obtain individual-specific markers of Indonesian coconut palms and their association. Coconut palms were selected randomly from 7 accessions (74 palms) and then evaluated based on 10 SSR primers and main agromorphological characters. This research was carried out during 2017 and 2018 at Plant Breeding Laboratory, UGM, Yogyakarta and Mapanget Experimental Garden, IPCRI, North Sulawesi. CnCir 123-300, CnCir 87-120, CnCir J2-205 were unique alleles that resulted in this study. Five of eleven specific alleles were associated with essential traits of dwarf coconut. CnCir123-250 and CnCir123-300 were specific alleles associated with almost all characters observed in this study. CnCirA9-100 is the only allele that is negatively associated with the height of 11 leaf scars and positively associated with the main characters. Therefore, it is advantageous as a marker-assisted selection of the current coconut palm breeding program. Applications of specific molecular markers associated with essential characters are expected to improve the sustainability of coconut breeding programs.

Keywords: specific individual alleles, dwarf coconut, association, SSR

1. Introduction

Coconut palm (Cocos nucifera L) is the only species in the Cocos genus in the family Areaceae [1]. Coconut palm is a non-cambial perennial crop that requires a longer time to implement plant breeding programs. Morphological markers play a pivotal role in all plant breeding programs [2]. By determining the allele of a DNA marker, plants that possess particular genes or quantitative trait loci (QTL) may be identified based on their genotype instead of their phenotype. Therefore, DNA markers that are tightly linked to target loci can be used as a substitute to assist phenotypic screening [3].

Developing and testing hybrid varieties of coconut is currently a significant field of research in many countries [4]. The use of morphological markers in assessing the purity test of plant breeding programs has many limitations. However, the morphological characters such as petiole color, germination rate, number of leaves in the seedlings, and fruit circumference at fruit mature ages can still be used in identifying the validity of the hybrid [5]. Analysis of petiole color in purity assessment has been seen to be less accurate [6]. These markers can only be utilized if both parents are homozygous for the yellow, red, or green characters, and have different colors [5, 7]. Morphological markers are challenging to apply
in genetic purity testing of hybrid coconut palms, but most hybrid coconuts have similar characteristics, making them difficult to evaluate [4].

Morphological markers take a pivotal role in the selection of essential traits in all plant breeding programs. However, morphological markers as genetic markers in coconut plant breeding programs have several obstacles: such as the influence of the environment and the phase of plant development that determines their expression. Thus, both result in an ineffective utilization due to a long vegetative phase [8, 9]. DNA markers that are tight-linked to target loci as a substitute for assist phenotype screening [3].

The development of molecular markers through individual identification has been used for protecting varieties, identify genotypes, strains, varieties, assess the purity of seeds, and solving the uncertainty parents in palm oil breeding [10]. Special alleles have been promising to be used as molecular markers to assess seed purity in coconut breeding. Information about specific alleles can improve the effectiveness and efficiency of coconut palms breeding as non-cambial perennial crops. Furthermore, specific alleles at several SSRs loci as a part of DNA fingerprinting can be used as specific identities (IDs) for local accession to complement morphological markers for the benefit of variety protection [11]. Unfortunately, information about specific alleles and their associations with essential characters on coconut palms has never been done in Indonesia. Therefore, this study aimed to obtain individual-specific markers of coconut palms in Indonesia, and its association with production, and the other essential traits.

2. Materials and method
The experiment was conducted from July 2017 to May 2018 by survey method in the Mapanget Experimental Garden, Indonesian Palm Crops Research Institute and Laboratory of Plant Breeding and Genetics, Faculty of Agriculture, Gadjah Mada University. Seventy-four individuals of coconut palms were selected randomly from 7 accessions of Dwarf coconut, germplasm collection in Mapanget Experimental Garden, Indonesian Palm Crops Research Institute, North Sulawesi, Indonesia (Table 1).

Table 1. Name of accessions, their code, province, and country of origin.

| Names of dwarf accessions | Accession code | Province/country of origin | Number of palms |
|---------------------------|----------------|----------------------------|-----------------|
| Sagerat Orange Dwarf      | SOD            | North Sulawesi             | 10              |
| Tebing Tinggi Dwarf       | TTD            | North Sumatera             | 15              |
| Jombang Green Dwarf       | JGD            | East Java                  | 15              |
| Bali Yellow Dwarf         | BYD            | Bali                       | 9               |
| Nias Green Dwarf          | NGD            | North Sumatera             | 10              |
| Nias Yellow Dwarf         | NYD            | North Sumatera             | 10              |
| Waingapu Red Dwarf        | WRD            | East Nusa Tenggara         | 5               |

Morphological observations were conducted on production characteristics based on the Manual on Standardized Research Technique in Coconut Breeding (STANTECH manual) COGENT [12]. Then, molecular analysis using SSR markers was performed on all of the samples.

2.1. DNA isolation, marker selection and DNA amplification by PCR
DNA isolation was performed using CTAB buffer [13] with PVP, CTAB, and mercaptoethanol concentration modification. This research uses CNZ 51, coconut SSR primers developed by [14], and nine other primers by [15] (Table 2).
Table 2. Primer name, oligonucleotide sequences, size of primer, and melting temperature (TM) used to amplify DNA of the eighth palm accessions.

| No | Primer name | Oligonucleotide sequences | Size (base) | TM (°C) |
|----|-------------|--------------------------|-------------|---------|
| 1  | CnCir 121   | F: TTGGTCTATTGCATTTTC    | 18          | 55      |
|    |             | R: TGGGATTGAGAGGT        |             |         |
| 2  | CnCir 2     | F: AGAACCTTGCTCCAC       | 16          | 55      |
|    |             | R: TCCAGCCATTCCATC       |             |         |
| 3  | CnCir 167   | F: GGTGGGTAAGTAACATC     | 18          | 57      |
|    |             | R: GTGATAACAGAACCCTC     |             |         |
| 4  | CnCir 123   | F: TACAGAGGACAAATTTD     | 20          | 46      |
|    |             | R: TAAAAATTTATAAGGTAAAA  |             |         |
| 5  | CnCir C9    | F: ATTTDTAGCTTCACATGAA   | 21          | 55      |
|    |             | R: TCAATTDCAAGAAGACCTTTG |             |         |
| 6  | CnCir C5    | F: CTGAAAGATATGGTTDTATGC | 20          | 52      |
|    |             | R: TTDDCCAGATTGATTD      |             |         |
| 7  | CnCir A9    | F: GGAACATGGTTDCTTTD     | 17          | 55      |
|    |             | R: CCTCTGAATCTGCGGG      |             |         |
| 8  | CnCir 87    | F: AACCAAGAATGAGTCG      | 19          | 55      |
|    |             | R: TTTGAACCTTCTCTATGGG   |             |         |
| 9  | CnCir J2    | F: CATTGTCAAGTTTDDTTT    | 20          | 52      |
|    |             | R: GTACACATCTCTCTCDTDC   |             |         |
| 10 | CNZ 51      | F: AAAGTGAAGTTGGAATATGG | 20          | 55      |
|    |             | R: AGAGAGGATCAGGTTGTDGT  |             |         |

Nucleotide base amplification was performed using PCR Bio-Rad T 100™Thermo Cycler machine and master mix PCR kit KAPA 2G FAST. The formula for each coconut DNA samples reaction consisted of 5 × 6.25 µl PCR buffer, MgCl2 25 mM 0.25 µl, nuclease-free water 2.35 µl, SSR marker (forward and reverse) 0.65 µl, and DNA working solution 2 µl. The total volume for each reaction was 11.5 µl. The PCR cycle begins at 95°C for 3 minutes as pre-PCR, followed by denaturation at 95°C for 15 seconds, annealing step 51-55°C for 15 seconds, 72°C elongation stage for 1 second, and the cycle is repeated 40 times. This PCR process ended with post PCR at a temperature of 72°C for 10 minutes. The PCR cycle in this research was based on Pesik [16].

2.2. PCR Products confirmation and data analysis
The DNA amplification products were confirmed by electrophoresis using 1.5% mixture gel (metaphor: agarose = 3 : 1) in TBE 1× buffer with 100 V, 400 mA for 60 minutes[17]. GelRed DNA staining was utilized to detect the position of alleles in the gel. Alleles were scored as a codominant marker. Association between specific individual alleles to production, fruit component and height of 11 leaf scars was performed with stepwise regression analysis with SAS 9.1 software[18, 19].

3. Result and discussion
The number of alleles produced by each primer varied between 3-7 with the percentage of polymorphic loci 37.21 % ± 5.18 % (Table 3). The number of alleles per locus depends on the diversity of germplasm accessions and the characteristics of the SSR markers. The most robust coconut microsatellite gene diversity was evidencing with CnCir C9 with a mean value of He 0.109 (Table 3).
Table 3. Number of alleles and level of gene diversity detected for 10 SSRs primer pairs.

| No | Locus name | Number of samples | Number of alleles | Gene diversity index (He) ± SD |
|----|------------|-------------------|------------------|-------------------------------|
| 1  | CnCir 121  | 74                | 4                | 0.140 ± 0.023                 |
| 2  | CnCir 2    | 74                | 7                | 0.111 ± 0.015                 |
| 3  | CnCir 167  | 74                | 6                | 0.108 ± 0.020                 |
| 4  | CnCir 123  | 74                | 3                | 0.033 ± 0.013                 |
| 5  | CnCir C9   | 74                | 3                | 0.215 ± 0.030                 |
| 6  | CnCir C5   | 74                | 4                | 0.151 ± 0.013                 |
| 7  | CnCir A9   | 74                | 4                | 0.027 ± 0.009                 |
| 8  | CnCir 87   | 74                | 3                | 0.061 ± 0.017                 |
| 9  | CnCir J2   | 74                | 4                | 0.107 ± 0.020                 |
| 10 | CNZ 51     | 74                | 4                | 0.121 ± 0.022                 |
|    | Mean± SD   |                   | 4.2± 1.3         | 0.109± 0.010                  |

Figure 1. Electrophoregram of PCR product amplified by CNZ 51 in GOS (1-5) and TTD (6-11) populations.

The number of produced alleles resulting from the amplification (Figure 1) of 10 SSR primers at each accession was found different, i.e., between 3 to 7 alleles. Local typical bands with at least 50% of the observed population vary in number from each accession, between 2 (NGD) to 8 alleles (TTD). According to [20], alleles are categorized as general alleles, namely if the frequency is ≥ 0.05 and the unique allele is <0.05. Some numbers of accession have unique alleles. Unique alleles are those with a relatively small frequency, less than a particular value such as 0.01 [21]. CnCir 123-300, CnCir 87-120, CnCir J2-205 were unique alleles that resulted in this study. These unique alleles are commonly called outlining alleles, and it quickly disappears from the gene pool when genetic deviations occur or because of the small population size [16]. The number of unique alleles for each accession is different. Unique alleles with relatively small frequency only possessed by some numbers in an accession are called specific alleles or private alleles.
Figure 2. Allelic patterns across populations in the dwarf coconut palm.

Based on this study, SOD, NGD, NYD, and WRD are accessions with no private alleles, while JGD has the highest number of private alleles (8). No accession-specific alleles were obtained in this research. Private alleles are unique alleles to a single population. Some of the private alleles are special alleles. The existence of private alleles can be used as a tool in coconut palms breeding programs, especially in hybrid seeds purity tests. Molecular markers have been used for variety identification and purity testing of hybrid seeds with high effectiveness and efficiency [22]. Furthermore, fingerprinting is a molecular technique that has been used to describe the molecular pattern of a genotype. Superior coconut palms which have private alleles might be used as a hybrid parent with fingerprinting identity. SSR are codominant markers, conserved and inherited. SSR marker is one of the best choices for DNA fingerprinting, especially for variety protection and identification of legitimate hybrids of intra varietal (Dwarf x Dwarf) with more excellent reliability [23].

Superior palms with unique genetic profiles can be selected as hybrid parents especially male parents (Table 4). Thus, all primers selected at the screening stage can be successfully used for hybrid seeds identification. It will be easier and more efficient to assess the validity of hybrid seeds when using specific alleles through the fingerprint method. The effectiveness of the selection method that encourages efficiency will directly increase the sustainability of the coconut seed production and breeding program.

| No | Accessions | No. of accessions | Primers | Alleles Size (bp) |
|----|------------|-------------------|---------|------------------|
| 1  | TTD        | 3, 4, 8           | CnCir 121 | 230              |
| 2  | BYD        | 2                 | CnCir J2  | 205              |
|    |            | 1, 9              | CnCir J2  | 220              |
| 3  | JGD        | 1, 10, 15         | CnCir 2  | 120              |
|    |            | 8                 | CnCir 2  | 150              |
|    |            | 8, 9, 10          | CnCir 2  | 220              |
|    |            | 7, 15             | CnCir 167| 350              |
|    |            | 3, 9, 10, 11      | CnCir 167| 400              |
|    |            | 14                | CnCir 123| 250              |
|    |            | 13                | CnCir 123| 300              |
|    |            | 7                 | CnCir 87  | 120              |
Production was the main goal in the coconut palm breeding program. This research shows that some of the alleles and specific alleles have a relationship with production characters (Table 5). Knowing molecular markers associated with productivity or other essential traits will give early information about the superior progeny. Furthermore, specific alleles possessed by the hybrid parent can be used as molecular markers to identify the legitimacy of seeds. Thus, superior individual-specific markers as a part of identity markers in genetic fingerprints have a high functional value. Therefore, molecular characterization of palm parent candidates as part of the seeds garden database is needed.

Based on this study, 3 primers (CnCir 167, CnCir 123 dan CnCirJ2) are associated with the number of leaves. Four in seven alleles associated with the number of leaves are specific alleles (Table 4). Moreover, CnCir 121, CnCir 2, CnCir 167, CnCir 123 and CNZ51 primers were positively associated with the number of fruits per bunch (Table 5). 3 specific alleles positively associated with the number of fruits per bunch, i.e CnCir121-230, CnCir2-120, and CnCir123-300. The results of this study are in line with the result of previous studies [16]. Moreover, [24] stated that CNZ 21 and CNZ 51 are associated with the fruits number per bunch. Based on [24], accession numbers on Mapanget Tall-32 that do not produce fruit until 11 years after planting do not have CNZ 21-270. There were nine alleles produced from five primers associated with the number of bunches, and 2 alleles of it were specific.

Table 5. Association between alleles and specific alleles of SSR with number of leaves and production characters of Dwarf coconut.

| No | Characters                | R²   | Alleles         | Specific Alleles | P-value | Association |
|----|--------------------------|------|-----------------|------------------|---------|-------------|
| 1  | Number of leaves         | 0.993| CnCir167-200    | 0.0128            | +       |
|    |                          |      | CnCir167-400    | ✓                 | 0.0006  | -           |
|    |                          |      | CnCir123-170    | <.0001            | +       |
|    |                          |      | CnCir123-250    | ✓                 | <.0001  | +           |
|    |                          |      | CnCir123-300    | ✓                 | <.0001  | +           |
|    |                          |      | CnCirJ2-220     | ✓                 | 0.0197  | -           |
|    |                          |      | CnCirJ2-220     | 0.002             | +       |
| 2  | Number of fruits/bunch   | 0.857| CnCir121-230    | 0.025             | +       |
|    |                          |      | CnCir2-120      | ✓                 | <.0001  | +           |
|    |                          |      | CnCir167-250    | 0.0128            | +       |
|    |                          |      | CnCir123-170    | <.0001            | +       |
|    |                          |      | CnCir123-300    | ✓                 | <.0001  | +           |
|    |                          |      | CNZ51-210       | 0.0018            | -       |
| 3  | Number of bunches        | 0.949| CnCir123-170    | <.0001            | +       |
|    |                          |      | CnCir123-250    | ✓                 | <.0001  | +           |
|    |                          |      | CnCir123-300    | ✓                 | <.0001  | +           |
|    |                          |      | CnCirC9-240     | 0.0075            | +       |
|    |                          |      | CNZ51-200       | 0.0436            | -       |
|    |                          |      | CnCirA9-80      | 0.0205            | -       |
|    |                          |      | CnCirC5-140     | 0.0009            | -       |
|    |                          |      | CnCirC5-150     | <.0001            | -       |
|    |                          |      | CnCirC5-175     | 0.0037            | -       |
The associations between molecular markers (SSR) and several fruit component characters are given in Table 5. In this study, several alleles were associated with almost all observed characters, such as CnCir123-250 and CnCir123-300 (Table 5, Table 6, and Table 7). Therefore, it is estimated, these alleles are associated with a gene which has pleiotropic effect.

**Table 6.** Association between alleles and specific alleles of SSR with fruit component characters of Dwarf coconut.

| No | Characters          | $R^2$   | Alleles          | Specific Alleles | P value | Association |
|----|---------------------|--------|------------------|------------------|---------|-------------|
| 1  | Weight of fruit     | 0.959  | CnCir121-200     |                  | 0.0160  | +           |
|    |                     |        | CnCir2-280       |                  | 0.0287  | -           |
|    |                     |        | CnCir167-250     |                  | 0.006   | -           |
|    |                     |        | CnCir123-170     | <.001            | +       |
|    |                     |        | CnCir123-250     | ✓                | <.001   | +           |
|    |                     |        | CnCir123-300     | ✓                | 0.0204  | +           |
|    |                     |        | CnCirC9-240      |                  | 0.032   | -           |
|    |                     |        | CnCirA9-100      | <.001            | +       |
|    |                     |        | CnCir87-180      |                  | 0.0324  | +           |
| 2  | Weight of dehusked fruit | 0.966 | CnCir2-120       | ✓                | 0.0338  | -           |
|    |                     |        | CnCir167-250     |                  | 0.0001  | -           |
|    |                     |        | CnCir123-170     | <.001            | +       |
|    |                     |        | CnCir123-250     | ✓                | <.001   | +           |
|    |                     |        | CnCir123-300     | ✓                | 0.0004  | +           |
|    |                     |        | CnCirC9-240      |                  | 0.004   | -           |
|    |                     |        | CnCirA9-100      | <.001            | +       |
|    |                     |        | CnCir87-180      |                  | 0.0293  | +           |
| 3  | Weight of endosperm | 0.979 | CnCir2-120       | ✓                | 0.0361  | -           |
|    |                     |        | CnCir167-250     | <.001            | -       |
|    |                     |        | CnCir123-170     | <.001            | -       |
|    |                     |        | CnCir123-250     | ✓                | <.001   | +           |
|    |                     |        | CnCir123-300     | ✓                | <.001   | +           |
|    |                     |        | CnCirA9-100      | <.001            | +       |
|    |                     |        | CnCir87-180      | <.001            | +       |
| 4  | Weight of coconut water | 0.852 | CnCir121-200     |                  | 0.0148  | +           |
|    |                     |        | CnCir167-250     |                  | 0.014   | -           |
|    |                     |        | CnCir123-170     |                  | 0.0026  | +           |
|    |                     |        | CnCir123-250     | ✓                | 0.0049  | +           |
|    |                     |        | CnCirC9-210      |                  | 0.0067  | +           |
|    |                     |        | CnCirC9-275      |                  | 0.0001  | +           |
|    |                     |        | CnCirA9-100      |                  | 0.0029  | +           |
| 5  | Endosperm thickness | 1     | CnCir123-170     | <.001            | +       |
|    |                     |        | CnCir123-250     | ✓                | <.001   | +           |
A low stem growth rate trait is one of the current breeding directions for coconut palms. Dwarf coconut is also often used as a female parent in hybrid development. Because of that, dwarf coconut palms with a low stem growth rate character will significantly assist pollinators in their work. Molecular markers that are negatively associated with 11 leave scars and positively associated with other essential characters, could be used in assisting coconut palms breeding programs (Table 6 and Table 5). CnCirA9-100 is the only allele in this study that is negatively associated with the height of 11 leaf scars and positively associated with the weight of fruit, the weight of dehusked fruit, the weight of endosperm, and the weight of coconut water.

| No | Character                              | R²  | Alleles     | Specific Alleles | P-value | Association |
|----|----------------------------------------|-----|-------------|------------------|---------|-------------|
| 1  | Height of 11 leaf scars                 | 0.986 | CnCir123-170 | <.0001          | +       |
|    |                                        |     | CnCir123-250 | ✓                | <.0001  | +           |
|    |                                        |     | CnCir123-300 | ✓                | <.0001  | +           |
|    |                                        |     | CnCirA9-100  | 0.0263           | -       |
|    |                                        |     | CnCir87-155  | <.0001           | +       |

4. Conclusion

Six of ten SSR primers produced specific alleles and the number of specific alleles for each accession is different. TTD, BYD, and JGD were three of seven accessions with specific alleles, while the highest number of specific alleles belong to JGD (8). Based on the results of this study, no specific accession alleles were obtained. Some alleles associated with production characters are also associated with other characters of fruit components. Five of eleven specific alleles were associated with essential traits of dwarf coconut. CnCir123-250 and CnCir123-300 were specific alleles associated with almost all characters. The allele, which is negatively associated with the height of 11 leaf scars and positively associated with the main characters (CnCirA9-100), is beneficial for marker-assisted selection to develop high productivity and low stem growth rate in coconut palms. The effectiveness of the selection method that encourages efficiency will directly increase the sustainability of the coconut seed production and breeding program.

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References

[1] Xiao Y, Luo Y, Yang Y, Fan H, Xia W, Mason A S, Zhao S, Sager R, Qiao F 2013 *Plant Omics Journal* 6 3 193-200
[2] Khalid M, Farhatullah, Khan N A, Din R, Khan M Y, Akmal M and Ali N 2010 *Pakistan Journal of Botany* 42 5 2995-3000
[3] Jonah P M, Bellp L L, Lucky O, Midau A and Moruppa S M 2011 *Global Journal of Science Frontier Research* 11 5 version 1.0 5-12
[4] Preethi P, Rajesh M K, Rahul C U, Jerard B A, Samsudeen K, Regi Jacob Thomas and Karun A2016 *Journal of Plantation Crop* 442 77-84
[5] Rajesh M K, Jerard B A, Preethi P, Thomas RJ and Karun A2014 *Crop Breeding and Applied Biotechnology* 14 1 36-41
[6] Perera L 2010*Int. J. Coconut R & D.* 26 1 39-43
[7] Batugal Pand and Bourdeix R 2005 Conventional coconut breeding *Coconut Genetic Resources* ed. Batugal P, Rao R V and Oliver J (Selangor: International Plant Genetic Resources Institute) pp. 251-67
[8] Staub J C, Serquen F C and McCreight J A 1997 *Genetic Res and Crop evolution* **4** 4 315-26
[9] Peterson A H, Tanksley S D and Sorrels M E 1991 *Adv. Agron.* **46** 39-90
[10] Tasma I M 2014 *Buletin Palma* **15** 1 1-13
[11] Lestari P, Risliawati A, Utami D W, Hidayatun N, Santoso T J and Chaerani 2016 *Jurnal Biologi Indonesia* **12** 2 219-29
[12] Santos G A, Batugal P A, Othman A, Baudouin L and Labouisse JP 1996 *Manual on standardized research techniques in coconut breeding* (Rome: IPGRI)
[13] Doyle J J and Doyle J L 1987 *Focus* **12** 13-15
[14] Rivera R 1999 *Genom* **42** 668-75
[15] CIRAD 2002 Coconut microsatellite kit *A Laboratory Manual* ed. Montpellier F R (France: CIRAD)
[16] Pesik A 2016 Keragaman genetik plasma nutfah kelapa Indonesia dan penentuan identitas kelapa hibrida berdasarkan marka molecular *Dissertation* IPB Bogor, Indonesia
[17] Kristamtini, Taryono, Basunanda P and Murti R H 2014 *Jurnal Agro Biogen* **10** 2 69-76
[18] Cahyaningrum P, Taryono and Rimbawanto A 2012 *Indonesian Journal of Biotechnology* **17** 161-68
[19] Aristya VE and Taryono 2016 *AIP Conf. Proc* vol **1755** issue 1 040007(Jogjakarta: Indonesia) p 1-6
[20] Marshall D and Brown A 1975 Optimum sampling strategies in genetic conservation. Crop Genetik Resources for Today and Tomorrow *Crop Genetic Resources for Today and Tomorrow* ed Frankel O H and Hawkes J G (Cambridge: Cambridge University Press) pp. 53-80
[21] Kimura M 1983 *Mol Biol Evol* **1** 84 – 93
[22] Ye-yun X, Zhan Z, Yi-ping X, Long-ping Y 2005 *Rice Science* **12** 1 7-12
[23] Azevedo A O N, Azevedo C D O, Santos P H A D, Ramos H C C, Boechat M S B, Arêdes F A S, Ramos S R R, Mirizola L A, Perera L, Aragão W M and Pereira M G 2018 *Crop Breeding and Applied Biotechnology* **18** 409-16
[24] Pandin D S 2009 *Perspektif* **9** 1 21-35